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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of )  
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Chin-Pin LIU, et al. )  
 )  
Serial No. 10/074,257 ) Examiner: Vandervegt, Francois P.  
 )  
Filed: February 14, 2002 ) Group Art Unit: 1644  
 )  
For: ANTIGEN SPECIFIC )  
RECOMBINANT MHC CLASS II ) Confirmation No. 5061  
MOLECULES AND METHOD OF )  
USE )

DECLARATION UNDER 37 C.F.R. §1.132

Dear Sir:

I, Chih-Pin Liu, do solemnly declare that:

1. I am the same Chih-Pin Liu listed as an inventor on the above-referenced patent application.
2. I received a B.S. in biochemistry from National Taiwan University, ROC in 1983, an M.S. in microbiology from the University of Minnesota in 1988, and a Ph.D. in immunology from the University of Wisconsin in 1991. I received post-doctoral training in the Division of Immunology at Beth Israel Hospital/Harvard Medical School in Boston, Massachusetts (1991-1993) and at the Howard Hughes Medical Institute, National Jewish Center for Immunology/Respiratory Medicine in Denver, Colorado (1994-1997). In 1998, I became an Assistant Professor in the Division of Immunology at the Beckman Research Institute of the City of Hope. I have

since been promoted to the rank of Associate Professor in the Division of Immunology at the Beckman Research Institute and hold a joint appointment at the Department of Diabetes, Endocrinology and Metabolism at the Beckman Research Institute. A copy of my curriculum vitae is attached herewith as Exhibit A.

3. I have reviewed and am familiar with U.S. Patent Application Serial No. 10/074,257, filed February 14, 2002, entitled "Antigen Specific Recombinant MHC Class II Molecules and Methods of Use", including the claims currently pending in the application. I also have reviewed and am familiar with the Office Action dated December 5, 2006 and the references cited therein.
4. The claims currently pending in the application are directed to stable glutamic acid decarboxylase (GAD)-peptide-specific Class II *MHC* complexes comprising (1) the extracellular portion of the  $\beta$  chain of a Class II *MHC* molecule selected from the group consisting of I-Ag7 and DQ, and (2) a GAD peptide that binds to said Class II *MHC* molecule. These complexes, commonly referred to as "tetramers" in the art, can be used as reagents which can identify and isolate T cells involved in autoimmune disease such as Type 1 diabetes.

5. In the outstanding Office Action, the examiner has asserted that it would be obvious to combine the Tisch et al. reference, which discusses administration of GAD peptides to NOD mice to inhibit diabetes development, with the Crawford et al. reference, which teaches soluble recombinant *MHC* Class II molecules with antigenic peptides attached to the beta chain, and the Altman et al. reference, which is not discussed in the Office Action. The Examiner has cited Tisch et al. for teaching that the GAD peptides were effective in inducing regulatory Th2 cells and Crawford for teaching the usefulness of multimeric constructs in identifying and tracking antigen-specific T cells as motivation for the combination, which assertedly would have a reasonable expectation of success. The examiner has rejected claims in this application based on grounds of obviousness over these three references.
6. I applied to the National Institutes of Health for a pilot grant to generate stable GAD-peptide-specific Class II *MHC* complexes, as claimed here, that would allow one to identify and isolate T cells involved in autoimmune disease (specifically Type 1 diabetes). The reviewers of this grant application expressed concern about the potential inherent instability of I-Ag7 heterodimers and the binding affinity of the GAD peptides. In summary, the grant reviewers


believed that there was a possibility that we would not succeed in generating stable peptide-specific Class II *MHC* complexes in general using the proposed approach for generating autoimmune disease-associated class II *MHC* genes containing autoantigenic peptides such as GAD peptides. The approach proposed in the grant proposal was the same as that described in the present application.

7. Therefore in my opinion, the art at the time this application was filed taught that discovering GAD peptides that bind to class II *MHC* molecules was unpredictable and risky. See Hackett and Sharma, *Nat. Immunol.* 3(10): 887-889, 2002 attached hereto as Exhibit B and of record in this case. This reference refers to a workshop held in June, 2002, after the filing date of this application, in which the technical problems in achieving workable, stable Class II multimers, particularly those containing autoantigenic peptides, were discussed. See Hackett, pages 887-888. In my opinion, this reference shows that the invention claimed here was not considered possible in June 2002 by skilled artisans.
8. Therefore, it is my opinion that the cited art and the art generally provided no expectation of success for the invention claimed in this application, much less a reasonable expectation of success.

9. In my opinion, ours is the first research group to generate such autoimmune disease-associated class II *MHC* complex reagents and to use them to isolate large numbers of autoreactive T cells. Since the time of filing of this patent application, other groups have generated several of the claimed reagents containing autoantigenic peptides and have been able to identify and isolate T cells specific for the autoantigens. See Stratmann et al., *J. Clin. Invest.* 112(6):902-914, 2003, attached hereto as Exhibit C, and Jang et al., *J. Immunol.* 171:4175-4186, 2003, attached hereto as Exhibit D.
10. It is my opinion that it would not have been obvious to achieve the invention claimed in the accompanying patent application because, among other reasons, the skilled person would not have had a reasonable expectation of success when combining any particular peptide, and certainly not GAD peptides with the class II *MHC* molecules described in our application.

11. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Chih-Pin Liu

  
\_\_\_\_\_  
Date 5-1-07

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## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.  
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Liu Chih-Pin	POSITION TITLE Associate Professor
eRA COMMONS USER NAME	

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
National Taiwan University, ROC	B.S.	1983	Biochemistry
University of Minnesota, Minneapolis, MN	M.S.	1988	Microbiology
University of Wisconsin, Madison, WI	Ph.D.	1991	Immunology

### A. Positions & Honors:

#### **Positions:**

2003 - present Associate Professor, Department of Diabetes, Endocrinology, and Metabolism, Beckman Research Institute, City of Hope, Duarte, CA

2003 - present Associate Professor, Division of Immunology, Beckman Research Institute, City of Hope, Duarte, CA

1998 - 2003 Assistant Professor, Division of Immunology, Beckman Research Institute, City of Hope, Duarte, CA.

1994 - 1997 Research Associate, Howard Hughes Medical Institute, National Jewish Center for Immunology/Respiratory Medicine, Denver, CO.

1991 - 1993 Research Fellow, Department of Medicine, Division of Immunology, Beth Israel Hospital/Harvard Medical School, Boston, MA.

1988 - 1991 Research Assistant, Department of Zoology, University of Wisconsin, Madison, WI.

1985 - 1988 Research Assistant, Department of Microbiology, University of Minnesota, Minneapolis, MN.

1983 - 1984 Research Assistant, Department of Agricultural Chemistry, National Taiwan University, Taiwan, R.O.C.

**Honors:** John Alexander, M.D. Research Award, American Heart Association (2005); Cancer Research Institute Postdoctoral Fellowship (1992-1994); Sigma Xi Graduate Student Research Award finalist, Wisconsin Chapter (1991); Jefferson Davis Fund Predoctoral Fellowship (1991); Jefferson Davis Fund Travel Award (1989).

### B. Selected Peer Reviewed Publications:

1. Liu C.-P., Bach F.H., and Wu S. 1988. Molecular studies of a rare DR2/LD-5a/DQw3 HLA class II haplotype: Multiple genetic mechanisms in the generation of polymorphic HLA class II genes. J. Immunol. 140:3631-3639.
2. Liu C.-P., Wu S., and Bach F.H. Molecular studies of a rare HLA haplotype: Implications for mechanisms of generating class II gene polymorphisms. In Dupont B. (ed.): Immunobiology of HLA, vol. II: Immunogenetics and Histocompatibility. Springer-Berlag, NY, 1989; pp. 194-197.
3. Wu S., Lu D., Madden M., Liu C.-P., Miyokawa N., Bach F.H., and Saunders T.L. 1990. Full-length DQ  $\beta$  cDNA sequences of HLA-DR2/DQw1 subtypes: Genetic interactions between two DQ  $\beta$  loci generate human class II HLA diversity. Human Immunol. 27:305-322.
4. Liu C.-P., and Auerbach R. 1991a. Ontogeny of murine T cells: Thymus regulated development of T cell receptor-bearing cells derived from embryonic yolk sac. Eur. J. Immunol. 21:1849-1855.

5. Liu C.-P., and Auerbach R. 1991b. In vitro development of murine T cells from pre-thymic and pre-liver embryonic yolk sac stem cells. *Development*. 113: 1315-1323.
6. Liu C.-P., Globerson A. and Auerbach R. 1993. A cloned lymphoid Thy1<sup>+</sup> tumor line derived from murine yolk sac cells maintained in long-term cell culture in the absence of a thymic microenvironment expresses an unusual cell surface phenotype. *Thymus*. 21:221-233.
7. Liu C.-P., Ueda R., She J., Sancho J., Wang B., Weddell G., Loring J., Kurahara C., Dudley E. C., Hayday A., Terhorst C., and Huang M. 1993. Abnormal T cell development in CD3 $\zeta^{-/-}$  mutant mice and identification of a novel T cell population in the intestine. *The EMBO J*. 12: 4863-4875.
8. Wang B., Levelt C., Salio M., Zheng D., Sancho J., Liu C.-P., She J., Huang M., Higgins K., Sunshine M.-J., Eichmann K., Lacy E., Lonberg N., and Terhorst C. 1995. Over-expression of CD3 $\epsilon$  transgenes blocks T lymphocyte development. *Internatl. Immunol*. 7: 435-448.
9. Liu C.-P., Kappler J., and Marrack, P. 1996. Thymocytes can become mature T cells without passing through the CD4<sup>+</sup>CD8<sup>+</sup> double positive stage. *J. Exp. Med*. 184: 1619-1630.
10. Marrack P., Ignatowicz, L., Parker, D., Liu, C.-P., and Kappler, J. 1996. The structure and specificity of T cells selected by a single MHC/peptide combination. In "HLA and Disease – The Molecular Basis". Alfred Benzen Symposium 40. Published by Munks Goard Copenhagen.
11. Liu C.-P., Lin, W.-J., Huang, M., Kappler J., and Marrack, P. 1997. Development and function of T cells in TCR/CD3  $\zeta$ -gene knockout mice expressing Fc $\epsilon$ R1 $\gamma$ . *Proc. Nat. Acad. Sci*. 94: 616-621.
12. She J., Simpson, S. J., Gupta, A., Hollander, G., Levelt, C., Liu, C.-P., Allen, D., van Houten, N., Wang, B., Terhorst, C. 1997. CD16-expressing CD8 $\alpha^+$  T lymphocytes in the intestinal epithelium: Possible precursors of Fc  $\gamma$  R<sup>+</sup>CD8 $\alpha^+$  T cells. *J. Immunol*. 158: 4678-4687.
13. Liu C.-P., Parker, D., Kappler J., and Marrack, P. 1997. Selection of antigen-specific T cells by a single IE<sup>k</sup>/peptide combination. *J. Exp. Med*. 186: 1441-1450.
14. Liu C.-P., Crawford, F., Marrack, P., and Kappler J. 1998. T cell positive selection by a high density, low affinity ligand. *Proc. Nat'l. Acad Sci. USA*, 95:4522-4526.
15. Liu, C.-P., Jiang, K., Wu, C.-H., W.-H. Lee, and Lin, W.-J. 2000. Detection of glutamic acid decarboxylase (GAD) activated T cells with I-A<sup>g7</sup> tetramers. *Proc. Nat'l Acad. Sci. USA*, 97:14596-14601.
16. Peng, Y., Liu, C.P. 2002. The cross-reactivity of I-A(g7) and I-A(d) tetramers. *Chin Med J (Engl)*. 2002. 115:1579-1581.
17. Peng Y, Liu, C.P. 2002. Characterization of proteolipid protein-peptide-specific CD(4)(+) T cell of experimental allergic encephalomyelitis in Biozzi AB/H mice. *Chin Med J (Engl)*. 2002. 115:521-524.
18. Lee, W.-H., Ramos, T., Krymskaya, L., and Liu, C.-P. 2003. Development of T cells expressing an altered TCR complex. *Eur. J. Immunol*. 33:2696-2705.
19. You, S., Chen, C., Lee, W.-H., Wu, C.-H., Judkowski, V., Pinilla, C., Wilson, D.B., and Liu, C.-P. 2003. Detection and characterization of T cells specific for BDC2.5 T cell-stimulating peptides. *J. Immunol*. 170:4011-4020.
20. Chen, C.L., Lee, W.-H., Pen, Y., Snow, P., Liu, C.-P. 2003. Induction of autoantigen-specific Th2 and Tr1 regulatory T cells and modulation of autoimmune diabetes. *J. Immunol*. 171:733-744.
21. You, S., Chen, C., Lee, W.-H., Brusko, T., Atkinson, M., and Liu, C.-P. 2004. Presence of diabetes-inhibiting glutamic acid decarboxylase-specific IL-10-dependent regulatory T cells in naive NOD mice. *J. Immunol*. 173:6777-6785
22. Zhong, L., Wu, C.-H., Lee, W.-H., and Liu, C.-P. 2004. ZAP-70, but not Syk, tyrosine kinase can mediate apoptosis of T cells through the Fas/FasL, caspase-8, and caspase-3 pathways. *J. Immunol*. 172:1472-1482.
23. Krymskaya, L., Lee, W.-H., Zhong, L., and Liu, C.-P. 2005. Polarized development of memory cell-like IFN- $\gamma$ -producing cells in the absence of TCR chain  $\zeta$ . *J. Immunol*. 174:1188-1195.



24. Zhou, J., Hinton, D.R., Stohlman, S.A., Liu, C.P., Zhong, L., Marten, N.W. 2005. Maintenance of CD8+ T cells during acute viral infection of the central nervous system requires CD4+ T cells but not interleukin-2. *Viral Immunol.* 18:162-169.
25. Chen, C.L., Lee, W.-H., Zhong, L., Liu, C.-P. 2006. Regulatory T cells can mediate their function through the stimulation of antigen-presenting cells to produce immunosuppressive nitric oxide. *J. Immunol.* 176:3449-3460.
26. Liu, C.-P. 2006. Glutamic acid decarboxylase-specific regulatory T cells. *Immunology of Diabetes IV: Progression in Understanding. Annals New York Acad. Sci.* 1079:161-170.
27. Lee, M.-H., Lee, W.-H., Contag, C., and Liu, C.-P. 2007. Non-CD4 splenocytes help diabetogenic CD4<sup>+</sup> T cells induce accelerated diabetes by promoting invasive insulinitis without altering their trafficking patterns in animals. Submitted.
28. Itakura, S., Asari, S., Rawson J., Ito, T., Liu, C.-P., Sasaki, N., Todorov, I., Kandeel, F., Mullen, Y. 2007. Mesenchymal stem cells facilitate induction of mixed chimerism and islet allograft tolerance without GVHD in the rat. *Am. J. Transplantation.* 7:336-346.
29. Asari, S., Itakura, S., Ferreri, K., Liu, C.-P., Kuroda, Y., Kandeel, F., and Mullen, Y. 2007. Mesenchymal stem cells prevent B cell terminal differentiation by the down-regulation of the Blimp-1 expression through humoral factors. Submitted.

### **C. Current Research Support:**

1. 0555066Y 7/1/05 - 6/30/07  
American Heart Association  
Title: Non-invasive *in vivo* imaging analyses of autoreactive T cells  
The major goal of the proposed studies is to develop novel bioluminescence methods to visualize T cell trafficking in animals.  
Role: P.I.
2. 1-06-RA-83 1/1/06 - 12/31/08  
American Diabetes Association  
Title: Induction of immune tolerance to pancreatic islets  
The major goal of the proposed studies is to induce immune tolerance to pancreatic islets.  
Role: P.I.
3. ADF-1481 4/1/06 - 3/31/08  
HL Snyder Medical Foundation  
Title: T cells and islet inflammation  
The major goal of the proposed studies is to study the role of T cell during islet inflammation.  
Role: P.I.
4. F31 CA117055 8/1/05 - 7/31/10  
National Institute of Health  
Title: The Kirschstein-NRSA Predoctoral Fellowship  
The major goal of this award is to provide support for a predoctoral student Serina Ortiz. Her project is to use proteomics approach to examine the phosphoproteome of T cells, and to identify and characterize proteins or kinases involved in the regulation of leukemic T cell growth.  
Role: Mentor/Sponsor
5. R01 CA122976 6/1/06 - 5/31/11  
Title: Role of STAT3 in tumor immune evasion and immune suppression

The major goal of the proposed study is to evaluate the role of STAT3 in T cells that regulate tumor immunity.

Role: Co-investigator (P.I.: H. Yu).

6. COH Lymphoma SPORE Development Research Award 7/1/06 - 6/31/07  
National Institute of Health  
Title: Proteomics analyses of proteins regulating activation and death of T lymphoma cells  
The goal is to identify signaling proteins that mediate the function of Syk kinase in suppressing the activation and activation induced cell death in T lymphoma cells.  
Role: P.I.
7. Excellence Award 12/1/06 - 11/30/08  
Beckman Research Institute  
Title: T cells and autoimmune-mediated diseases  
This award is supporting the PI to investigate the role of antigen-specific regulatory T cells in the regulation of autoimmune-mediated diseases.  
Role: P.I.
8. The Norris Foundation Innovative Research Award 10/1/06 – 9/30/07  
Title: Epigenetic control of regulatory and pathogenic T cells  
The award is to support studies that examine how the function of regulatory and pathogenic T cells are controlled at the genetic levels.  
Role: P.I.

### **Completed Research Support**

1. R01 AI44143 3/1/99 - 2/28/04  
National Institute of Health  
Title: Influence of TCR/CD3 complex on T cell development  
The major goals of these studies are to determine how the development and function of T cells may be regulated by signals mediated through the TCR/CD3 complexes that contain normal and altered components.  
Role: P.I.
2. R21 DK60190 9/30/01 - 9/29/03  
National Institute of Health  
Title: Regulation of Type 1 Diabetes Using Ribozymes  
The major goal of this application was to design ribozyme-based gene therapy approach to modulate the function of autoreactive T cells.  
Role: P.I.
3. R21 AI44429 9/30/98 - 9/29/00  
National Institute of Health  
Title: Regulatory Mechanisms in Type 1 Diabetes  
The major goal of the proposed study was to understand the mechanisms by which the interaction of MHC/self-peptide complex with T cell antigen receptor may regulate the development of autoimmune diseases.  
Role: P.I.
4. U19 AI050864 7/1/05 - 6/30/06  
National Institute of Health  
Title: Isolation and characterization of antigen-specific CD4<sup>+</sup> T regulatory cells

The major goal of the proposed studies is to isolate and characterize CD4<sup>+</sup> T regulatory cells specific for major autoantigens.

Role: Subcontract P.I. (P.I.: G. Eisenbarth)

5. R01 AI48847 2/1/01 - 1/31/07

National Institute of Health

Title: Regulatory mechanisms in type 1 diabetes

The major goal of the proposed studies is to isolate autoantigen-specific T cells and evaluate their roles in regulating type 1 diabetes.

Role: P.I.

Although tetramer technology has been wildly successful for examination of MHC class I-recognizing T cells, the same hasn't been true for MHC class II reagents. A recent workshop at the US National Institute of Allergy and Infectious Diseases was convened to address this.

## Frontiers in peptide-MHC class II multimer technology

Charles J. Hackett<sup>1</sup> and Opendra K. Sharma<sup>2</sup>

Divisions of <sup>1</sup>Allergy, Immunology and Transplantation and <sup>2</sup>Acquired Immunodeficiency Syndrome, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20817, USA. (ch187q@nih.gov and os4g@nih.gov).

The capability to analyze antigen-specific CD4<sup>+</sup> T cells in single-cell detail offers the potential for enormous advances in every area of research involving immune responses and immune homeostasis. In-depth characterization of major histocompatibility complex (MHC) class II-restricted T cells is important in such diverse undertakings as the design and testing of vaccines, monitoring therapy for autoimmune diseases, HIV research, organ transplantation and devising strategies to treat T cell-mediated hypersensitivities such as certain antibiotic allergies and poison ivy rashes. However, fishing out individual T cells, each specific for a particular peptide-MHC (pMHC) molecule combination, requires a very specialized set of hooks.

Davis, McHeyzer-Williams and Altman established a practical design for the molecular tools that tag T cells in an antigen-specific manner in 1996<sup>1</sup>. Most familiarly known as "tetramers," four identical biotin-containing pMHC complexes are attached to fluorescently labeled avidin for binding to specific T cell receptors (TCRs) (Fig. 1). Tetramers, or as alternatives, pMHC multimers based on the immunoglobulin G (IgG) backbone<sup>2</sup>, have rapidly become the gold standard for T cell analyses and manipulation, enabling the enumeration, isolation, stimulation or targeting of substances to T cells of known antigen specificity<sup>3</sup>. In addition to basic research objectives, pMHC multimers have potential as diagnostic and treatment modalities, including the selection of cells for adoptive transfer<sup>4,5</sup> (T. Bruneau, New York, NY; G. Nepom, Seattle, WA).

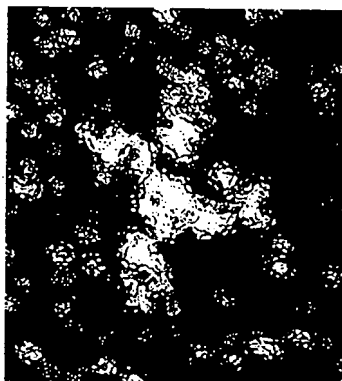
The basic concept of pMHC multimers is simple: clonal T cells can be identified with a multivalent form of the specific complex of antigenic pMHC that is their cognate ligand. The engineered form of the pMHC complex provides specificity, and the Ig or tetramer construct offers increased avidity: this compensates for the relatively low affinity that is characteristic of monomeric pMHC-TCR interactions. Although MHC molecules come in two basic forms, MHC class I and II—which are recognized by CD8<sup>+</sup> cytotoxic T cells and CD4<sup>+</sup> helper T cells, respectively—most tetramers produced so far have been class I MHC-based reagents. Nevertheless, the very first tetramer made by Davis and coworkers was based on a class II MHC molecule, and a number of different class II reagents have led to important research findings in murine and human systems<sup>6,7</sup>. Although production of MHC class II-based reagents is not yet

routine, many problems are now being overcome. These include the engineering of stable protein constructs, reliable production of recombinant class II MHC proteins in insect cell lines and the use of covalently attached peptides to overcome expression and external peptide-loading problems for certain alleles and peptide combinations<sup>6,8</sup>. However, the remaining problems—which include issues of TCR avidity and CD4<sup>+</sup> T cell frequency—have created rough terrain, if not roadblocks, that hinder the widespread application of class II MHC tetramers.

A workshop convened on 18–19 June 2002 by the National Institute of Allergy and Infectious Diseases at the National Institutes of Health in Bethesda, MD, addressed major technical issues surrounding the production and use of pMHC class II multimers. The consensus emerged that whereas many pMHC multimers are already proving to be effective tools for the analysis and enumeration of MHC class II-restricted CD4<sup>+</sup> T cells, determining exactly why others do not work remains an inexact science at present. Continued improvements in reagent design and specially tailored strategies for their application will emerge from basic studies of TCR-MHC interactions as well as from understanding lifestyle differences between CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

The class I MHC transmembrane protein consists of a single heavy chain that contains the complete peptide-binding groove and is stable in the soluble form complexed with its essentially invariant light chain  $\beta_2$ -microglobulin. In contrast, the peptide-binding site of MHC class II has contributions from both of its two membrane-anchored chains. When not associated with the cell membrane or complexed with a particularly high-affinity peptide, these chains tend to dissociate, so most tetramer designers engineer molecular

"zippers" to keep the class II MHC chains together. More significantly, the MHC class I peptide-binding groove has closed ends that define and limit the size of peptides that can bind, whereas the open-ended groove of the MHC class II molecule accommodates core peptides with flanking regions of considerably different lengths. In addition, the peptide-binding pockets of the class II molecule seem to be less stringent in their preference for particular amino acid side chains. Even well defined peptides may bind to the same MHC class II molecule in two or more frames, producing conformational ambiguity that



**Figure 1. Electron micrograph of a class I MHC tetramer.** Tetramers based on attachment of biotinylated pMHC monomers are excellent reagents for CD8<sup>+</sup> T cells, but the class II counterparts do not perform with the same consistency on CD4<sup>+</sup> T cells. The high rigidity of the tetramer arms may be a liability for such reagents in the class II system. (Courtesy of L. Teyton).

undermines the specificity of a distinct molecular reagent. Thus, it can be very difficult to define peptide-binding motifs because a class II molecule can elicit an array of T cells with related, but distinct, antigen specificities<sup>9</sup>. Recreating that complexity in tetramer reagents is a challenge specific to the MHC class II system.

Are there also fundamental differences between MHC class I and II molecules in the strength of their binding interactions with peptides or TCRs? Binding studies of peptides with isolated class II MHC molecules indicate that, at least for certain strong-binding peptides, the  $IC_{50}$  (concentration needed to competitively inhibit 50% of the binding of a standard peptide) can be in the nanomolar range (L. Teyton, La Jolla, CA), similar to other reported pMHC class I and II interactions<sup>10,11</sup>. Dissociation constants of TCR–MHC class II interactions in the range of  $10 \times 10^{-6} - 35 \times 10^{-6}$  M (L. Stern, Cambridge, MA; E. Ward, Dallas, TX) are also similar to those reported for MHC class I–TCR binding<sup>12</sup>. There remains some disagreement as to whether T cells stain better with tetramer reagents at higher temperatures that permit metabolic internalization of TCR or at lower temperatures that reflect the entropic barriers to stable binding. But different practical applications need pMHC class II reagents to function at both higher and lower temperatures—at 37 °C to drive proliferation or to anergize T cells (D. Busch, Munich, Germany; T. Brumeanu; G. Nepom) and at cooler temperatures to stain cells in frozen tissue sections (L. Teyton)—so a single condition will not fit all applications. Stronger affinities of particular peptides for their MHC-presenting element results in more stable

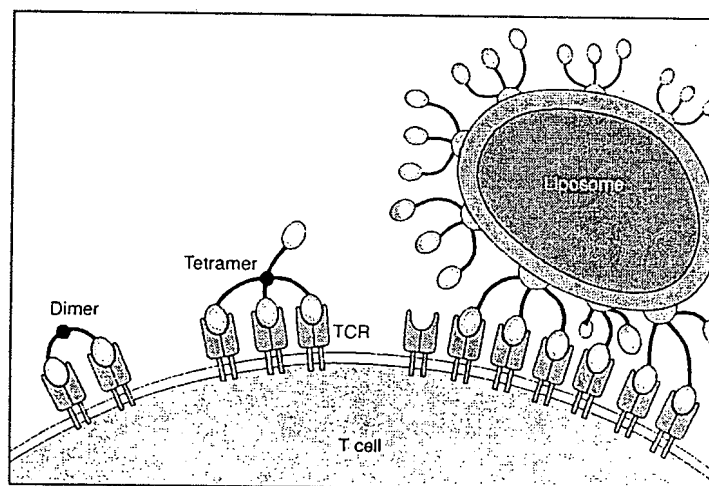
tetramers. Thus, engineering approaches that would maximize pMHC affinity as well as improve pMHC–TCR affinity without affecting specificity could result in better tetramers.

However, certain important types of TCR–MHC class II interactions may tend to cluster at the weaker end of the MHC–TCR affinity spectrum. One broad category in which some suspect that low-strength pMHC–TCR interactions may predominate is the autoantigens, perhaps related to their escape from thymic selection through low-avidity interactions<sup>13</sup>. Autoreactive T cells have been difficult to find with class II multimers in several human autoimmune diseases and animal models, including diabetes, Lyme arthritis, autoimmune gastritis and multiple sclerosis (D. Hafler, Boston, MA; C. Liu, Duarte, CA; K. Wucherpfennig, Boston, MA; W. Kwok, Seattle, WA; D. Margulies, Bethesda, MD; G. Nepom; T. Brumeanu).

More clear is that low avidity only compounds another fundamental problem: CD4<sup>+</sup> T cells of a given antigen specificity seem to be less abundant than their CD8<sup>+</sup> counterparts. This may be because peptides from some antigens are generated at low efficiency, which results in low density on antigen-presenting cells, or it might reflect localized

tissue-specific expression. Locating antigen-specific T cells with tetramers is largely a numbers game: it is difficult to detect cells at a frequency lower than 0.2%. Whereas CD8<sup>+</sup> T cells expand to large numbers in response to microbial infection or vaccination<sup>14</sup> and maintain high numbers for prolonged periods of time, there is no such surge for CD4<sup>+</sup> cells<sup>6</sup>. CD4<sup>+</sup> tetramer-binding cell frequencies in the blood or normal lymphoid tissues are in the range of 1 in 30,000. Greater numbers of antigen-specific CD4<sup>+</sup> T cells can be found in the tissues affected by autoimmune diseases or in an infection. But presently, the most practical approach for detecting CD4<sup>+</sup> T cells is *ex vivo* antigen-driven expansion of the T cells, after which one back-calculates the precursor frequencies. Lymphocytes labeled with the fluorescent molecule carboxyfluorescein diacetate succinimidyl diester (CFSE) distribute the dye evenly to daughter cells, permitting calculation of the number of divisions in the tetramer-binding cells. Calculation of a CD4<sup>+</sup> T cell precursor frequency of 1 in 30,000 after seven *in vitro* divisions is feasible (G. Nepom). Despite the promise of this approach, the difficulty of

meaningful interpretation of numbers extrapolated from days 7 or 10 back to day 0, and how these evaluations can be applied practically in clinical settings, remains a complex task. One alternative, explored in the enumeration of MHC class II-restricted HIV antigens in the peripheral blood, is to exploit high-throughput fluorescence analysis to count low frequency events with higher statistical precision (R. Sekaly, Montreal, Canada). Peripheral mononuclear blood cells sampled from patients during primary HIV infection showed as many as 1.5% CD4<sup>+</sup> T cells stained at 37 °C by an individual tetramer (B. Yassine-Diab, Montreal, Canada). Also, there is a need to explore other body



**Figure 2. Flexible multivalent pMHC class II reagents.** Dimers based on IgG as well as higher order multimers produced with cross-linking molecules more flexible than avidin may have distinct advantages for binding TCR on CD4<sup>+</sup> T cells. Liposomes with pMHC molecules clustered in microdomains are promising to become important reagents for further study.

tissues and fluids, besides peripheral blood, that may contain distinctive populations of CD4<sup>+</sup> T cells: for example, T cells at the site of inflammation may be of greater frequency or higher affinity than peripheral T cells (D. Hafler).

What if pMHC class II reagents could be devised that bind strongly to specific CD4<sup>+</sup> T cells, stain them intensely and have insignificant nonspecific background binding—wouldn't that make up for weak avidity and low frequency? The prospects are promising. pMHC tetramers based on the avidin scaffold are rigid molecules that may not engage multiple TCRs freely<sup>15</sup>. The immunoglobulin hinge region, perhaps an overlooked feature of pMHC reagents based on the IgG framework, may in fact play a key role in allowing strong simultaneous binding to multiple TCR molecules (J. Schneek, Baltimore, MD; N. Glaichenhaus, Valbonne, France; K. Wucherpfennig; T. Brumeanu). Also, chromatographic analyses of many "tetramer" preparations indicate that considerable aggregation may inadvertently be present that could contribute significantly to binding results (L. Stern).

Nonspecific background binding is frequently the limiting factor in approaches based on multivalency, but exciting new observations that

well constructed membrane arrays of pMHC class II complexes may overcome that problem potentially open up a new type of reagent for studying T helper cells. Artificial membrane spheres—liposomes—that incorporate the murine MHC class II complex I-A<sup>b</sup> complexed with an ovalbumin peptide strongly and specifically stain T cell lines (Fig. 2). Liposomes outperformed tetramers in the detection of CD4<sup>+</sup> cells taken directly from lymphocytic choriomeningitis virus-infected mice; with an irrelevant peptide, liposomes served as a negative control (L. Teyton). Much manipulation is possible in the liposome system, including pMHC incorporation (as monomer, trimer or tetramer), the lipid composition, liposome size and the fluorescent label. Liposomal “artificial antigen-presenting cells” have also been devised that mimic the high-density micromembrane domains observed in the immunological synapse<sup>16</sup> (S. Albani, San Diego, CA). With this approach, liposomes that focused HLA DR\*1101 complexed with an influenza hemagglutinin peptide into lipid domains with biotinylated cholera toxin inserted in the bilayer were successful in detecting human polyclonal antigen-specific T cells after a relatively short (72-h) expansion *in vitro*. T cell detection was better with the high-density domains than with randomly distributed class II MHC complexes in liposomes. Like tetramers, liposomes can be used to stain T cells at room temperature or lower, whereas under culture conditions at 37 °C, they stimulate T cells. Other pMHC multimers also can trigger T cells, but liposomes may offer a platform for incorporating costimulatory molecules or other ligands and/or receptors to finely control T cell responses.

In many cases, researchers must be able to remove the pMHC reagents after their use in functional isolation of T cell populations. Leaving the multimers attached under culture conditions or adoptive transfer *in vivo* may lead to abnormal cell activation, anergy or unwanted effects of T cell activation on bystander cells induced by TCR cross-linking. A recently reported new technology allows for reversible removal of pMHC reagents from cell surface<sup>17</sup>. This approach replaces biotinylation of class I MHC molecules with a peptide sequence fused to the COOH terminus of  $\beta_2$ -microglobulin that binds a mutated streptavidin molecule. Addition of free d-biotin results in rapid dissociation of surface-bound class I MHC multimers from the cell surface. Whether this technology will work for dissociation of class II multimers is under investigation (D. Busch). Liposomes containing MHC multimers were removed with 200 mM imidazole (L. Teyton).

These are early days for liposomes as antigen-specific reagents for CD4<sup>+</sup> T cells. More studies are needed to determine whether the

low backgrounds and high binding are typical. However, improved modeling of TCR-MHC multimeric binding<sup>18</sup>, further understanding of increased antigen sensitivity by activated T cells<sup>19</sup> and more refined information about the immunological synapse—the site at which T cells interact with their targets—should support the development of reagents that match well the recognition needs of CD4<sup>+</sup> T cells. Future research needs to define how closely one must mimic the target to increase the number of catches on these molecular hooks.

Reagent resources, such as the NIH Tetramer Facility (<http://www.niaid.nih.gov/reposit/tetramer/index.html>), can promote MHC class II-based reagent design, refinement and application. Contributions of the NIH Tetramer Facility include providing standardized reagents to a wide range of investigators, facilitating collaborative studies, identifying appropriate positive and negative controls and serving as a clearinghouse for protocols and comparative binding data (J. Altman and J. Lippolis, Atlanta, GA). The NIH Tetramer Facility resources are freely available to all investigators, regardless of whether they have NIH funding. The Facility has supplied over 1400 class I MHC tetramers to researchers worldwide. Investigators in all areas of immunology research should avail themselves of the Facility's new class II MHC reagent offerings and help pioneer the widespread application and development of these important immunology tools.

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It has been demonstrated that expression of class II MHC gene is a major genetic factor determining susceptibility to the disease. These genes also select a population of pathogenic or regulatory T cells specific for various autoantigens that are involved in the development of various autoimmune disease. Therefore, being able to have such T cells for further molecular and cellular studies help us understand the pathogenic and regulatory mechanisms underlying the disease process. However, identification of such T cells has been extremely difficult. The ability of making class II MHC tetramers significantly help us to address those problems. Nevertheless, it has not been easy to generate such reagents. In fact, when we applied to NIH for a pilot grant application to generate class II MHC tetramers that allow us to identify and isolate T cells involved in autoimmune disease (specifically type 1 diabetes), the reviewers of the application thought that the proposed approaches were novel and could be highly rewarding if it works. However, the reviewer also thought that the proposed approaches were highly risky and they did not expect that we would be successful. They did not think that, using the approach we proposed in the grant application, we would succeed in generating tetramers using autoimmune disease-associated class II MHC genes containing autoantigenic peptides.

However, it turned out that we are the first research group that can not only generate such autoimmune disease-associated class II MHC tetramer but also use such tetramer to isolate a large number of autoreactive T cells. Since then, we have generated several such tetramers containing autoantigenic peptides and were able to identify and isolate T cells specific for the autoantigens.

# Susceptible MHC alleles, not background genes, select an autoimmune T cell reactivity

See the related Commentary beginning on page 826.

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To detect and characterize autoreactive T cells in diabetes-prone NOD mice, we have developed a multimeric MHC reagent with high affinity for the BDC-2.5 T cell receptor, which is reactive against a pancreatic autoantigen. A distinct population of T cells is detected in NOD mice that recognizes the same MHC/peptide target. These T cells are positively selected in the thymus at a surprisingly high frequency and exported to the periphery. They are activated specifically in the pancreatic LNs, demonstrating an autoimmune specificity that recapitulates that of the BDC-2.5 cell. These phenomena are also observed in mouse lines that share with NOD the H-2<sup>b</sup> MHC haplotype but carry diabetes-resistance background genes. Thus, a susceptible haplotype at the MHC seems to be the only element required for the selection and emergence of autoreactive T cells, without requiring other diabetogenic loci from the NOD genome.

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## Introduction

Type 1 diabetes, or IDDM, results from the destruction of pancreatic islet  $\beta$  cells by a complex autoimmune process to which both genetic and environmental factors contribute (1–3). In humans, as in model NOD mice, genetic control is complex: the main genetic contribution to susceptibility resides in class II loci of the MHC, with particular sequences and structures in haplotypes that confer susceptibility in NOD and in diabetic patients (4–8). In mice, as in humans, a complex assortment of other susceptibility “background” genes that affect immune function also contribute to diabetes risk (3, 9, 10). IDDM is marked by the presence and activation of autoreactive T cells, whose origin remains a puzzle. It is not clear whether they result

from a failure of central or of peripheral tolerance, and whether MHC susceptibility alleles or background genes play a determining role in their selection. It has been suggested, for example, that the particular MHC-II alleles of the NOD mouse, because they bind peptides with peculiar kinetics or specificity, may inefficiently affect negative selection of autoreactive cells (11–13). Others have suggested that the background genes of the NOD mouse lead to poor negative selection and central-tolerance induction (14, 15). Finally, it is not clear whether the disease in NOD mice depends on a central-tolerance defect that lets a large number of high-affinity cells escape, or on a peripheral incapacity to control their aggressiveness (15–17). Transgenic models have shown that, for the most part, T cells reactive against peripheral antigens are selected in the thymus much like other T cells are (18–21), without the imprints of negative selection that mark T cell populations reactive against antigens expressed in the thymus (22–25). Yet these analyses only yield examples of the fates of individual T cell receptors (TCRs), reflecting a single affinity for self. It would be desirable to track broader populations, but the analysis has suffered from the absence of tools that would allow, in nontransgenic animals, side-by-side comparison of T cell repertoires between susceptible and resistant individuals. The advent of MHC multimer

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**Nonstandard abbreviations used:** T cell receptor (TCR); glucose-6-phosphate isomerase peptide 282–292 (GPI); phycoerythrin (PE); pancreatic lymph node (PLN); hen-egg lysozyme (HEL); resonance unit (RU); medium fluorescence intensity (MFI).



reagents, with which TCRs of particular specificities can be detected, now offers this possibility (26, 27).

Several autoreactive T cell clones isolated from diabetic NOD mice have been shown to be pathogenic in transfer experiments (28–33). BDC-2.5 is the best characterized of these clones. BDC-2.5 was derived from a diabetic female NOD mouse and was shown to greatly accelerate disease when transferred to young animals (30–32). This CD4<sup>+</sup> Th1 clone displays a V $\beta$ 4/V $\alpha$ 1 (AV1S5) TCR heterodimer (34). BDC-2.5 is restricted by A $\beta$ 7, the lone MHC class II molecule of NOD mice (4, 5). The nature of the presented antigen is still unknown, although it has been shown to be associated with the membrane fraction of  $\beta$  granules (35). Mice transgenic for the BDC-2.5 TCR show a robust positive selection of CD4<sup>+</sup> cells in the thymus. These are then exported, naive and fully reactive, to peripheral lymphoid organs. Infiltration of the pancreatic islets is precocious and synchronized (18). It seems, in this transgenic mouse, that the regulatory genes (36), molecules (37), cells (36), or intercellular milieu (38, 39) that modulate the progression to diabetes all act at a peripheral level, affecting the damage wrought there by the BDC-2.5 T cells, rather than the thymic control of their maturation.

We decided to use the BDC-2.5 system to test directly for the presence of autoreactive T cells, in nontransgenic animals, that might share the same antigenic specificity. Thus, an MHC-mimetic peptide with high agonistic activity for BDC-2.5 T cells (40, 41) was complexed to the A $\beta$ 7 molecule in an MHC multimer molecule (26, 27). Tetramers of this A $\beta$ 7/mimotope complex did prove specific for BDC-2.5 T cells. Strikingly, in NOD mice, they labeled a “natural” A $\beta$ 7/BDC-2.5 mimotope-reactive population, whose fate we could track. This report demonstrates the unique role of the MHC susceptibility genes in selecting this specificity.

## Methods

**Antibodies, hybridomas, and T cell lines.** T cell hybridomas BDC-2.5 and R28 have been described elsewhere and were maintained in RPMI-1640/10% FCS (30, 42). BDC T cell clones were maintained in culture by successive rounds of stimulation with  $\beta$  cell granule membranes from  $\beta$  cell tumors as a source of antigen and  $\gamma$ -irradiated NOD splenocytes as APCs as described previously (31, 35). T cell clones were restimulated with  $\beta$  cell granule membrane protein for 4 days and expanded in the presence of IL-2 prior to staining. All antibodies mentioned in this study were obtained from BD Pharmingen (San Diego, California, USA). V $\beta$  TCR-specific antibodies were directed against V $\beta$ 's 2, 3, 4, 5.1, 5.2, 6, 7, 8.1, 8.2, 8.3, 9, 10, 11, 12, 13, 14, and 17, respectively.

**Mouse strains and immunization.** NOD/LtJ, NOR/LtJ, C57BL/6, BALB/c, BDC-2.5/N, RAG<sup>0/0</sup>/NOD (18, 43), and KRN TCR transgenic mice that recognize glucose-6-phosphate isomerase peptide 282–292 (hereafter referred to as GPI) in the context of A $\beta$ 7 MHC molecules (23, 44) were either purchased from The Jackson Labo-

ratory (Bar Harbor, Maine, USA) or bred in our special pathogen-free animal facility (Joslin Diabetes Center and The Scripps Research Institute). For in vivo T cell response studies, mice were immunized in the base of the tail and in footpads with 10  $\mu$ g of BDC-2.5 mimotope peptide reconstituted in PBS and emulsified in CFA. Animals were sacrificed 8 days after immunization.

**Construction, expression, and purification of BDC-2.5 TCR and A $\beta$ 7/peptide molecules; generation of tetrameric MHC molecules.** The cDNA for the  $\alpha$  and  $\beta$  chains of the BDC-2.5 TCR was obtained by RT-PCR using Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) according to the manufacturer's instructions, and RNA was isolated from BDC-2.5 T cells and R28 transgenic T cells. Oligo-dT primers and sequence-specific primers (V $\alpha$ 1 [AV1S5], 5'-AAAAA-GAATTCGGTACCGAAATGCATTCCTTACATGTTTCAC-3'; V $\beta$ 4, 5'-AAAAAATATAGGTACCGAATTCGGAATGGGCTC-CATTTTCCTCAG-3'; C $\alpha$ , 5'-AAAAATAAGAATTCGGTACCGAAATGCATTCCTTACATGTTTCACTAGTG-3'; and C $\beta$ , 3'-TTTATTTTGTGCGACTCAACTGGACCACAGCCT-CAGCGT-5') were used for cDNA synthesis and PCR amplification, respectively. PCR products were subcloned into pCR2.1-TOPO cloning vector (Invitrogen Corp., Carlsbad, California, USA), sequenced, and subcloned into the metallothionein promoter-based fly expression vector pRMHa3 (45). Each of the final constructs coded for the  $\alpha$ 1 $\alpha$ 2 and the  $\beta$ 1 $\beta$ 2 domains, respectively, followed by a linker sequence (SSADL), a thrombin site (LVPRGS), a leucine zipper (acidic for the  $\alpha$  chain, basic for the  $\beta$  chain) (46), and a hexahistidine tag. Vectors were transfected into SC2 cells, and stable cell lines and clones were established. Soluble TCRs were purified from culture supernatants as described previously (47). The generation of A $\beta$ 7/peptide complexes has been previously reported in detail (6, 13). The A $\beta$ 7/2.5mi and A $\beta$ 7/GPI molecules bear the sequences AHHPIWARMDA and LSIALHVGFDDH, respectively. The biotinylation sequence no. 85 from Schatz (48) follows the acidic zipper on the  $\alpha$  chain. Biotinylation of purified molecules was performed according to the manufacturer's instructions (13). Biotinylation was measured by immunodepletion on streptavidin-agarose beads and subsequent analysis by SDS-PAGE and scanning. Biotinylated molecules were tetramerized at 4°C overnight using phycoerythrin-labeled (PE-labeled) or APC-labeled streptavidin (BioSource International Inc., Camarillo, California, USA; and ProZyme Inc., San Leandro, California, USA), with a 5:1 molar ratio of biotinylated molecules to labeled streptavidin.

**Cell preparation, cell staining, flow cytometry analysis, and adoptive transfer.** Single-cell suspensions were prepared by mechanical disruption of the corresponding organs in HBSS buffer, and erythrocytes were removed by lysis. Cells were washed in FACS buffer (PBS containing 2% FCS and 0.04% NaN<sub>3</sub>) and incubated with 0.5 mg/ml of streptavidin and Fc block in FACS buffer for 1 hour at room temperature. Cells were then washed once and

stained with PE-labeled MHC/peptide tetramers at a final concentration of 10 µg/ml in FACS buffer for 1 hour at room temperature. For costaining of surface markers, APC-anti-CD4, FITC-anti-CD3, FITC-anti-CD44, PerCP-anti-B220, and PerCP-anti-CD8 from BD Pharmingen were used, as well as PE-Cy7-anti-CD8 and PE-Texas red-anti-CD45R (B220) from Caltag Laboratories Inc. (Burlingame, California, USA). Exclusion of dead cells was done by addition of 0.1 µg/ml of Hoechst 33342 (Molecular Probes Inc., Eugene, Oregon, USA) or propidium iodide. Flow cytometry was performed using a FACSCalibur instrument (Becton Dickinson Immunocytometry Systems, Mountain View, California, USA) at The Scripps Research Institute or a MoFlo instrument (Cytomation Inc., Fort Collins, Colorado, USA) at the Joslin Diabetes Center, and the data were analyzed using either CellQuest software (Becton Dickinson Immunocytometry Systems) or Summit software (Cytomation Inc.). Staining of T cell hybridomas and T cell clones with MHC tetramers was carried out in the same way. For cell transfer experiments, NOD splenocytes were stained the same way, sorted on a high-speed cell sorter, and injected intravenously into 4-week-old RAG<sup>0/0</sup>/NOD mice (with no irradiation).

**Generation of T cell hybridomas.** Lymphocytes were isolated either from pancreatic lymph nodes (PLNs) of naive 8-week-old NOD females or from popliteal LNs 8 days after immunization in tail and footpads with 2.5mi peptide in CFA. Cells were stimulated with concanavalin A for 3 days and subsequently fused with BW5147αβ<sup>-</sup> lymphoma cells. Hybridomas were stained with 2.5mi tetramer, and single cells were cloned by FACS.

**T cell activation assay.** Ninety-six-well flat-bottomed plates (Corning Inc., Corning, New York, USA) were coated with serially diluted MHC/peptide complexes in PBS overnight at 4°C and washed three times with PBS. T cell hybridomas were washed twice in PBS, resuspended in complete DMEM, and added at 5 × 10<sup>4</sup> cells per well in 200 µl, for 24 hours at 37°C. Alternatively, hybridomas were incubated with β cell granule membranes from β cell tumors as a source of antigen, or with purified pancreatic islets in the presence of γ-irradiated NOD splenocytes as APCs. Supernatants were harvested, and IL-2 production was determined using the IL-2-dependent cell line NK.

**Surface plasmon resonance.** A Biacore 2000 instrument (Biacore Inc., Piscataway, New Jersey, USA) was used to determine interactions between purified MHC/peptide complexes and TCR molecules. BDC-2.5 TCR was immobilized by amine-coupling chemistry on a CM5 research-grade sensor chip. Surface densities for individual experiments are indicated in Table 1. Injections of MHC/peptide molecules at the appropriate concentrations were performed in filtered and degassed PBS at a flow rate of 20 µl/min. In all experiments, A87/GPI MHC molecules were used as negative control. K<sub>d</sub> values as well as on and off rates were obtained by nonlin-

ear curve fitting of subtracted curves using the Langmuir 1:1 binding model with the BIAevaluation program (version 3.0.2; Biacore Inc.) and the global-fitting software Clamp (version 3.3) (49). The equilibrium K<sub>d</sub>, under steady-state conditions, was also determined using the BIAevaluation program.

**Immunocytochemistry.** Immunocytochemistry was performed essentially as previously described (50). Briefly, organs of interest were removed, embedded in Tissue-Tek OCT compound (Sakura Finetek USA Inc., Torrance, California, USA), and immediately frozen on dry ice. Sections of 6 µm thickness were cut at -16°C on a microtome (Cryocut 1800; Reichert-Jung, Leica Microsystems, Bannockburn, Illinois, USA). Staining was performed at 4°C. Sections were incubated with APC-labeled A87 tetramers (5 µg/ml in PBS containing 2% FCS) and a rat anti-CD4 antibody (L3T4) for 15 hours. Sections were then washed briefly with PBS and fixed with paraformaldehyde. After 3 hours of incubation with a rabbit anti-APC antibody (Biomedica Corp., Foster City, California, USA), the signal was amplified by staining with a Rhodamine Red-X-conjugated goat anti-rat antibody and a Cy5-labeled goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA). Insulin was detected on paraformaldehyde-fixed sections by successive incubation with a guinea pig anti-insulin antibody (DAKO Corp., Carpinteria, California, USA) and a FITC-conjugated anti-guinea pig antibody. Staining was visualized using a Bio-Rad MRC1024 laser scanning confocal microscope (Bio-Rad, Hercules, California, USA) fitted with a krypton/argon mixed-gas laser (excitation at 488 nm, 568 nm, and 647 nm) using a ×40 oil objective.

## Results

**Production of functional BDC-2.5 TCR and A87/2.5 mimotope complexes.** A surrogate ligand for BDC-2.5 T cells was isolated from a chemically synthesized random-peptide library based on its ability to stimulate BDC-2.5 T cells (41). We hypothesized that a strong agonistic peptide selected solely on the basis of its biological activity, without prior regard to the affinity of the peptide/MHC complex for the TCR, would be the optimum ligand to construct MHC class II tetramers. The peptide AHHPIWARMDA, hereafter referred to as BDC-2.5 mimotope (2.5mi), was isolated and optimized using this strategy. A fusion MHC β chain/2.5mi peptide was engineered as previously reported (6, 13). Similar molecules with A87-binding peptides from glucose-6-phosphate isomerase (<sub>282</sub>LSIALHVGFDH<sub>292</sub>, hereafter referred to as GPI) and hen-egg lysozyme (<sub>11</sub>AMKRHGLDNYRGYSL<sub>25</sub>, hereafter referred to as HEL) were also produced. The A87/GPI complex is recognized by the R28/KRN TCR (44, 51). The A<sup>4</sup> α chain cDNA was modified at its 3' end to harbor an acidic zipper sequence followed by the biotinylation sequence no. 85 from Schatz (48) and a histidine tag (46). Purified molecules were biotinylated using the BirA enzyme and repurified by size exclusion chromatography (Fig-

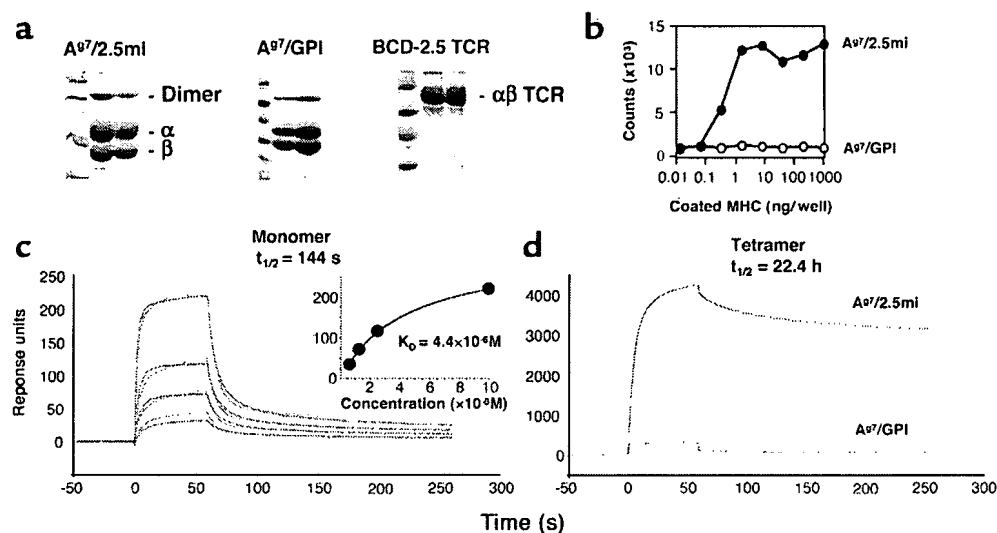
ure 1a). A<sup>87</sup>/2.5mi MHC molecules were functional when coated onto plates, stimulating BDC-2.5 hybridoma cells to secrete IL-2 (Figure 1b). Control A<sup>87</sup>/GPI molecules had no such effect (but did stimulate the R28 hybridoma; data not shown). In parallel, the  $\alpha$  and  $\beta$  chains of the BDC-2.5 TCR were cloned by RT-PCR from thymocytes of BDC-2.5 transgenic mice and modified to produce zippered soluble recombinant TCR (47) (Figure 1a).

The interaction between the recombinant BDC-2.5 TCR and A<sup>87</sup>/2.5mi MHC molecules was measured by surface plasmon resonance using A<sup>87</sup>/GPI MHC molecules as a negative control. Association and dissociation rates were calculated by global fitting from subtracted sensorgrams (Figure 1c and Table 1). The calculated  $K_d$  was approximately 0.5  $\mu$ M, whereas equilibrium data gave a value of 4.4  $\mu$ M at 25°C. These values were relatively low compared with those for most TCR/MHC class II agonistic-peptide interactions (52), suggesting that the mimotope is a very strong agonistic peptide for the BDC-2.5 TCR in the context of A<sup>87</sup>. Measurements at 37°C showed a slight decrease of calculated  $K_d$  to approximately 1.5  $\mu$ M (data not shown). The same MHC molecules were tetramerized with streptavidin and tetramers purified by gel filtration. Over a high-density surface ( $\sim$ 8,000 resonance units [RU]) of immobilized BDC-2.5 TCR, A<sup>87</sup>/GPI tetramers exhibited no binding, whereas A<sup>87</sup>/2.5mi tetramers showed specific binding and an increase of half-life to 22.4 hours compared with 144 seconds for the monomers (Figure 1, c and d). These results suggest that the interaction

between the A<sup>87</sup>/2.5mi tetramer and the BDC-2.5 TCR is long lasting. The affinity of A<sup>87</sup>/GPI molecules for their cognate TCR was also measured by surface plasmon resonance using recombinant soluble TCR and was evaluated to be approximately 1  $\mu$ M (T. Stratmann and L. Teyton, unpublished observations).

*A<sup>87</sup>/2.5mi tetramers are specific for the BDC-2.5 TCR.* A<sup>87</sup>/2.5mi and A<sup>87</sup>/GPI molecules were then tetramerized with streptavidin-phycoerythrin conjugate and the specificity of these reagents was tested against a panel of T cells. First, we stained T cell hybridomas for flow cytometry. As expected, A<sup>87</sup>/2.5mi multimers stained the BDC-2.5 but not the control hybridoma (Figure 2a), whereas A<sup>87</sup>/GPI multimers stained only R28 cells (data not shown).

Second, we tested the original BDC-2.5 T cell clone, as well as five other NOD-derived clones that are all specific for  $\beta$  cell antigens (31, 35) (Figure 2b). A<sup>87</sup>/2.5mi multimers stained specifically both BDC-2.5 and BDC-5.10 T cells, but not any of the other clones. This observation was consistent with the ability of the BDC-2.5 mimotope peptide to stimulate both these clones to produce IFN- $\gamma$  (data not shown). Finally, we tested our reagent with primary T cells from transgenic BDC-2.5 mice or another A<sup>87</sup>-restricted TCR transgenic mouse, KRN (23). The staining of naive T cells with MHC class II multimers has been a consistent hurdle for these reagents, and, in our hands at least, most of them failed even though they could stain hybridomas of similar specificity. When splenocytes from a BDC-2.5 trans-



**Figure 1**

Biophysical and functional characterization of A<sup>87</sup>/2.5mi and A<sup>87</sup>/GPI MHC molecules. (a) SDS-PAGE analysis of the various recombinant MHC and TCR molecules used in this study. Molecules were purified from culture supernatants of transfected *Drosophila melanogaster* cells. The peak fractions of the final size exclusion chromatography are shown. (b) Recombinant A<sup>87</sup>/2.5mi molecules can activate the BDC-2.5 T cell hybridoma. A<sup>87</sup>/2.5mi MHC monomers were coated at the indicated concentrations into 96-well plates. IL-2 production was measured from the supernatants after 24 hours of culture. (c) Surface plasmon resonance analysis of the A<sup>87</sup>/2.5mi MHC/BDC-2.5 TCR interaction. Left: BDC-2.5 TCR molecules were randomly immobilized on a CM5 chip, and A<sup>87</sup>/2.5mi or A<sup>87</sup>/GPI (negative control) MHC molecules were flown over the surface. Subtracted curves are shown in blue lines, calculated curves in red. A Langmuir 1:1 binding model was used for analysis. Concentrations of injected MHC molecules were 10  $\mu$ M, 2.5  $\mu$ M, 1.25  $\mu$ M, and 0.625  $\mu$ M. The inset shows a steady-state analysis of the same interaction. (d) Tetramers of A<sup>87</sup> with either peptide were flown over a high-density BDC-2.5 TCR surface. Unsubtracted curves corresponding to a tetramer concentration of 1  $\mu$ M are presented.

**Table 1**Kinetic constants from the interaction of BDC-2.5 TCR with A<sup>B</sup>/2.5mi MHC complexes

Experiment	$k_{on}$ ( $M^{-1}s^{-1}$ )	$k_{off}$ ( $s^{-1}$ )	$K_D$ (nM)
1	$0.8 \times 10^4$	$5.1 \times 10^{-3}$	650
2	$1.17 \times 10^4$	$5.9 \times 10^{-3}$	505
3	$1.07 \times 10^4$	$3.5 \times 10^{-3}$	330
Average $\pm$ SD	$1.01 \times 10^4 \pm 0.19 \times 10^4$	$4.83 \times 10^{-3} \pm 1.22 \times 10^{-3}$	$494 \pm 160$

Constants were obtained by surface plasmon resonance analysis. Surface densities of immobilized BDC-2.5 TCR for experiments 1, 2, and 3 were 895 RU, 447 RU, and 747 RU, respectively.  $K_D$  values were calculated from the ratio of kinetic constants ( $k_{off}/k_{on}$ ).

genic animal were stained with A<sup>B</sup>/2.5mi multimers and analyzed by FACS, 79.0% of the CD4<sup>+</sup> T cells were positive for the A<sup>B</sup>/2.5mi multimers (Figure 2c), consistent with the known frequency of clonotype expression in these mice (N. Martin-Orozco, unpublished observations). The same reagent was unable to stain T cells from the KRN transgenic mouse used as a control (Figure 2c). With the nontransgenic NOD control, the staining was also largely absent, but we were intrigued by a small, but clear, population of CD4<sup>+</sup> T cells that stained with the same intensity as in the BDC-2.5 transgenic mouse (Figure 2c).

**BDC-2.5-like T cells in the NOD mouse.** Several experiments were performed to ascertain the relevance of the small population of CD4<sup>+</sup> T cells that stained with the A<sup>B</sup>/2.5mi multimer (hereafter referred to as 2.5mi<sup>+</sup> T cells). The 2.5mi<sup>+</sup> T cells were alive, judging from scatter and vital dye exclusion, and expressed normal levels of CD4 and CD3 (not shown). The staining appeared specific, since it was not observed with control multimers loaded with GPI or HEL peptides (Figure 3a), which gave frequencies close to the background level (around 0.05% in most of our experiments). No CD8<sup>+</sup> T cells of similar specificity were identified (not shown). The genetic control provided by B6 mice (A<sup>b</sup> haplotype) showed that the 2.5mi<sup>+</sup> T cells were indeed specific to NOD mice (Figure 3a). Dual-label experiments were also performed, with two tetramer reagents added together. The 2.5mi<sup>+</sup> T cells did not bind the GPI tetramer simultaneously, arguing against nonspecific “sticking” of the reagents (Figure 3b). To verify the responsiveness of these cells, NOD mice were injected subcutaneously with the mimotope peptide in adjuvant. Eight days after immunization, 2.5mi<sup>+</sup> T cells increased in frequency from

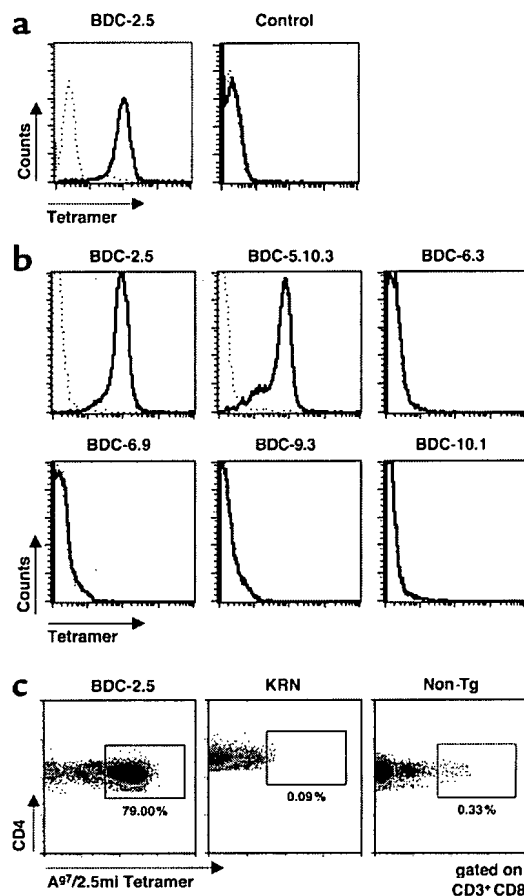
about 0.2% to 3.7% in the draining LNs (Figure 3c), demonstrating that the 2.5mi<sup>+</sup> T cell population was not anergized but responsive to antigenic challenge. To further demonstrate the specificity of A<sup>B</sup>/2.5mi tetramer staining, we tested the capacity of 2.5mi<sup>+</sup>CD4<sup>+</sup> cells to proliferate in response to homeostatic cues, after transfer into lymphopenic RAG<sup>0/0</sup>/NOD mice. As shown in Figure 3d, 2.5mi<sup>+</sup> T cells

expanded significantly in these recipients, forming a very distinct population among LN cells 20 days after transfer. Insulinitic attack was noted in one of the recipients, but not in all (data not shown), consistent with the requirement for CD8<sup>+</sup> cells, and conversely the blockade by other CD4<sup>+</sup> cells, in the development of autoimmunity in BDC-2.5 transgenic mice (18, 36).

To more directly visualize these results, in situ staining was carried out with MHC multimers and anti-CD4 antibodies. To date, only a few reports have mentioned using MHC class I tetramers for immunocytochemical staining (50, 53–55), and, to our knowledge, none has used MHC class II multimers. A technique for frozen nonfixed tissues was developed (50), and antibody- and tetramer-stained sections were analyzed by confocal microscopy (costained with anti-CD4 to identify T

**Figure 2**

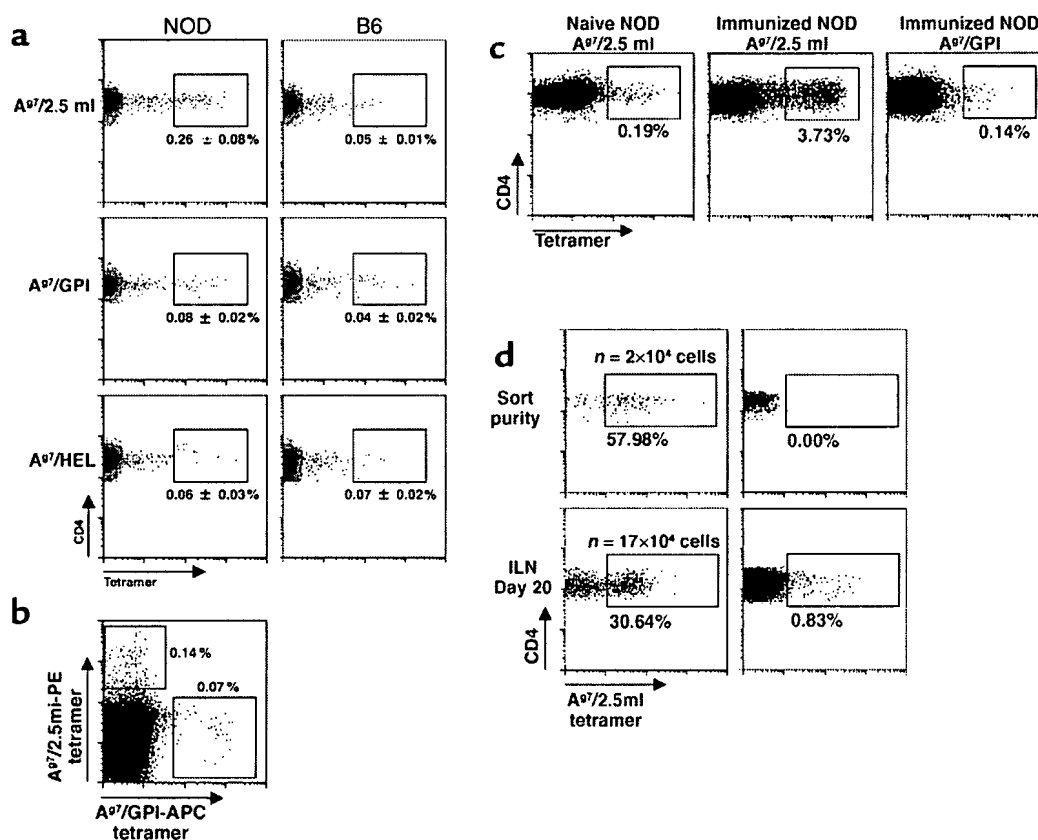
Specific recognition of 2.5mi<sup>+</sup> T cells by A<sup>B</sup>/2.5mi multimers. (a) BDC-2.5 T cell hybridoma and 19.2B T cell hybridoma (specific for glutamic acid decarboxylase peptide 282–292) were labeled with either A<sup>B</sup>/2.5mi tetramers (solid line) or A<sup>B</sup>/GPI tetramers (dotted line). (b) Staining of T cell clones specific for  $\beta$  cell granule membranes with A<sup>B</sup>/2.5mi tetramers. After 4 days of stimulation in vitro with islet-membrane preparations, BDC clones were stained with A<sup>B</sup>/2.5mi tetramers. (c) Inguinal LN cells from young adult BDC-2.5/NOD, KRN, and NOD (Non-Tg) mice were stained for CD4 and A<sup>B</sup>/2.5mi tetramers.



cells). In spleen or pancreas sections from BDC-2.5 transgenic mice, clear staining with the A<sup>g</sup>/2.5mi multimer could be identified, coincident with anti-CD4 staining (Supplemental Figure 1, <http://www.jci.org/cgi/content/full/112/6/902/DC1>). In sections from nontransgenic NOD mice, 2.5mi<sup>+</sup> T cells were also identified in situ by immunocytochemistry (Figure 4). Typically, we found between one and two 2.5mi<sup>+</sup> T cells per T cell zone in the spleen (not shown). Interestingly, they were also seen in PLNs and in islets in the infiltrating CD4<sup>+</sup> T cell population (Figure 4). Thus, by all these criteria, the prediabetic NOD mouse contains in its lymphoid organs a small but clearly identifiable population of 2.5mi<sup>+</sup> T cells that stains specifically with the A<sup>g</sup>/2.5mi reagent, is responsive to antigenic stimulation, and localizes to the expected organs.

**Life history of the 2.5mi<sup>+</sup> T cell population.** The dynamics of CD4<sup>+</sup>/2.5mi<sup>+</sup> T cells were characterized further by

FACS analysis. A representative experiment is shown in Figure 5, a and b, and compiled data are shown in Figure 5c. 2.5mi<sup>+</sup> T cells were clearly detectable among mature CD4<sup>+</sup>CD8<sup>-</sup>CD3<sup>hi</sup> thymocytes (Figure 5a) but not among CD4<sup>+</sup>CD8<sup>+</sup>CD3<sup>hi</sup> cells, as might be expected. These cells colonize the peripheral lymphoid organs: CD4<sup>+</sup> T cells in the spleen and subcutaneous LNs also showed clear staining above background, indicating that 2.5mi<sup>+</sup> T cells are exported and survive efficiently in the peripheral immune system (indeed, expand slightly, when thymus and LN frequencies are compared; Figure 5c). This proportion increased further in the LNs of the pancreatic group (PLNs) and among pancreas-infiltrating cells, suggesting that they were expanding in response to antigen. The expansion of BDC-2.5-like CD4<sup>+</sup> T cells in the pancreas has also been recently observed by You and colleagues using a very similar mimotope/A<sup>g</sup> molecule to identify those cells



**Figure 3**

Presence of 2.5mi<sup>+</sup> T cells in NOD mice. (a) Specific 2.5mi<sup>+</sup>CD4<sup>+</sup> T cells were identified in subcutaneous LNs from young adult NOD but not from B6 mice. Control stains for either A<sup>g</sup>/GPI or I-A<sup>g</sup>/HEL tetramers are shown in the lower panels. (b) Lack of cross-reactivity of A<sup>g</sup>/2.5mi tetramers with nonspecific T cells. Splenocytes from a NOD mouse were stained with PE-labeled A<sup>g</sup>/2.5mi tetramers, followed by incubation with APC-labeled A<sup>g</sup>/GPI tetramers. T cells were only stained by either of the tetramers, not by both simultaneously, indicating specific recognition. (c) 2.5mi<sup>+</sup>CD4<sup>+</sup> T cells expansion after immunization with the 2.5mi<sup>+</sup> peptide. Popliteal LN cells from nonimmunized and immunized NOD mice were stained with either A<sup>g</sup>/2.5mi or A<sup>g</sup>/GPI tetramers. (d) Expansion of 2.5mi<sup>+</sup>CD4<sup>+</sup> cells in a lymphopenic mouse reconstituted with 2.5mi<sup>+</sup>CD4<sup>+</sup> cells (or 2.5mi<sup>+</sup>CD4<sup>+</sup> cells as control), sorted from splenocytes of 5-week-old NOD mice and immediately analyzed for sort purity. Twenty thousand sorted cells were injected into RAG<sup>0/0</sup>/NOD mice. Inguinal LN cells were stained 20 days after transfer for the presence of 2.5mi<sup>+</sup>CD4<sup>+</sup> cells. The number of injected cells was not corrected for the presence of nonviable cells and is therefore likely to be overestimated. The number of 2.5mi<sup>+</sup>CD4<sup>+</sup> cells present in the whole mouse (n) was extrapolated to a full lymphoid compartment from the number of 2.5mi<sup>+</sup>CD4<sup>+</sup> cells observed in the inguinal LN by flow cytometry (taking into account multiple losses during the staining procedures and the flow cytometry acquisition itself).

**Table 2**  
Summary of the T cell hybridomas generated for this study from NOD female mice

Source of T cells	Total lines/ clones analyzed	Stained by A $\beta$ /2.5mi tetramer	Reactive with coated A $\beta$ /2.5mi molecules <sup>A</sup>	Tetramer <sup>+</sup> and reactive with coated MHC	Reactive with islets
NOD females, immunized	80	38	39	22	1 <sup>B</sup>
NOD females, naive	99	40	25 <sup>C</sup>	25	6 <sup>D</sup>

<sup>A</sup>A $\beta$ /2.5mi molecules were coated at 1  $\mu$ g/ml for these experiments. <sup>B</sup>Out of four subclones tested. <sup>C</sup>Out of 25 clones tested. <sup>D</sup>Out of 14 clones tested.

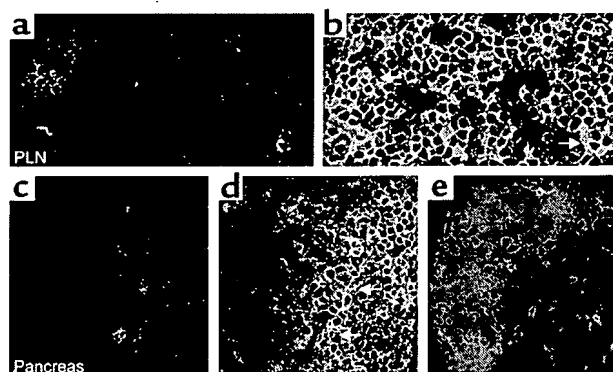
(56). This expansion and accumulation in the PLN and pancreas was suggestive of activation of the 2.5mi<sup>+</sup> T cell population by pancreatic antigens, similar to that of BDC-2.5 transgenic mice. This was confirmed by examination of T cell activation markers in the CD4 population and its 2.5mi<sup>+</sup> T cell subset. As shown in Figure 5d, an increased proportion of CD44<sup>hi</sup> cells was found among 2.5mi<sup>+</sup> T cells in the PLNs of young NOD mice, mirroring BDC-2.5 transgenics. This difference was not observed in bulk CD4<sup>+</sup> T cells of the same mice (10%  $\pm$  2% and 12%  $\pm$  2% of CD44<sup>hi</sup> cells in inguinal LN and PLN, respectively). This hallmark of activation was detected, to a lesser extent, in the neighboring superior mesenteric LNs, into which some drainage of the pancreas likewise occurs, but not in the spleen or other LNs unrelated to the pancreas (axillary, iliac, renal, and lower mesenteric), whereas almost no 2.5mi<sup>+</sup> cells were detected in Peyer's patches (data not shown). These data indicate that the 2.5mi<sup>+</sup> T cells do share the autoimmune specificity of the BDC-2.5 clonotype.

We were curious whether the percentage of 2.5mi<sup>+</sup> T cells in the CD4<sup>+</sup> T cell population increases with age in NOD females, paralleling the development of insulinitis and diabetes. PLNs and iliac LNs were removed from animals ranging from 2 to 16 weeks of age (four animals combined per time point), and the CD4 T cell population was analyzed for 2.5mi<sup>+</sup> T cells. Frequencies of 2.5mi<sup>+</sup> T cells varied from 0.05% to 0.15% over the course of the experiment (Figure 6). In the PLN, cells were found as early as week 2, and their frequency increased to peak at week 3 or 4. In the iliac LNs, 2.5mi<sup>+</sup> cells appeared later and climbed to a steady level from week 4 to week 16. The early wave of expansion in the PLN suggests site-specific activation and is also reminiscent of the 3- to 4-week checkpoint 1 that has been described in the 2.5 transgenic mouse (57). The control GPI-reactive cells remained at background level in both locations for the entire period of the experiment (Figure 6).

To further address the islet reactivity of these T cells, we immunized NOD female mice by injection of 2.5mi peptide into the footpads and generated hybridomas from T cells isolated from the draining LNs. A total of 120 hybridoma lines were generated, of which 80 were analyzed for their reactivity to coated peptide/MHC complexes and to free peptide in the presence of APCs, as well as by FACS analysis for staining with 2.5mi tetramers (Table 2, Table 3, and Figure 7a). About half of the lines were negative in both assays. Out of 39 and 38 lines, respectively, that tested positive for either reac-

tivity toward coated peptide/MHC complexes or staining by FACS, 22 clones were positive in both assays. With three exceptions, there was a clear positive correlation between IL-2 secretion and intensity of tetramer staining (data not shown). Of the remaining clones, about half tested positive in the activation assay, whereas the other half were only positive in the FACS analysis. However, staining by FACS of these clones was very low (medium fluorescence intensity [MFI] < 10 vs. 7 for the negative control). Double-positive lines were tested for islet reactivity. Of those clones, only one (clone A162.7) tested clearly positive (Table 3 and Figure 7c).

However, immunization with peptide could lead to the preferential selection of T cell specificities distinct from the fine specificity inherent to the original BDC-2.5 clone. To better understand the natural 2.5mi<sup>+</sup> T cell population, we generated another set of T cell hybridomas, from PLNs of naive animals. One hundred fifty-three lines were generated, and tetramer-positive cells were sorted by FACS and cloned. Ninety-nine clones were generated in such a way and reanalyzed by FACS for staining with the 2.5mi tetramer. Of these clones, 40 tested positive, with MFIs ranging from 17 to 138, indicating the heterogeneity of these clones in terms of avidity. (MFI > 10 defined positive clones, and MFI < 2 defined negative clones. TCR expression was identical for all clones.) Twenty-five of these clones were analyzed for IL-2 secretion in an activation assay using coated peptide/MHC complexes (Figure 7b). All clones positive for tetramer staining secreted



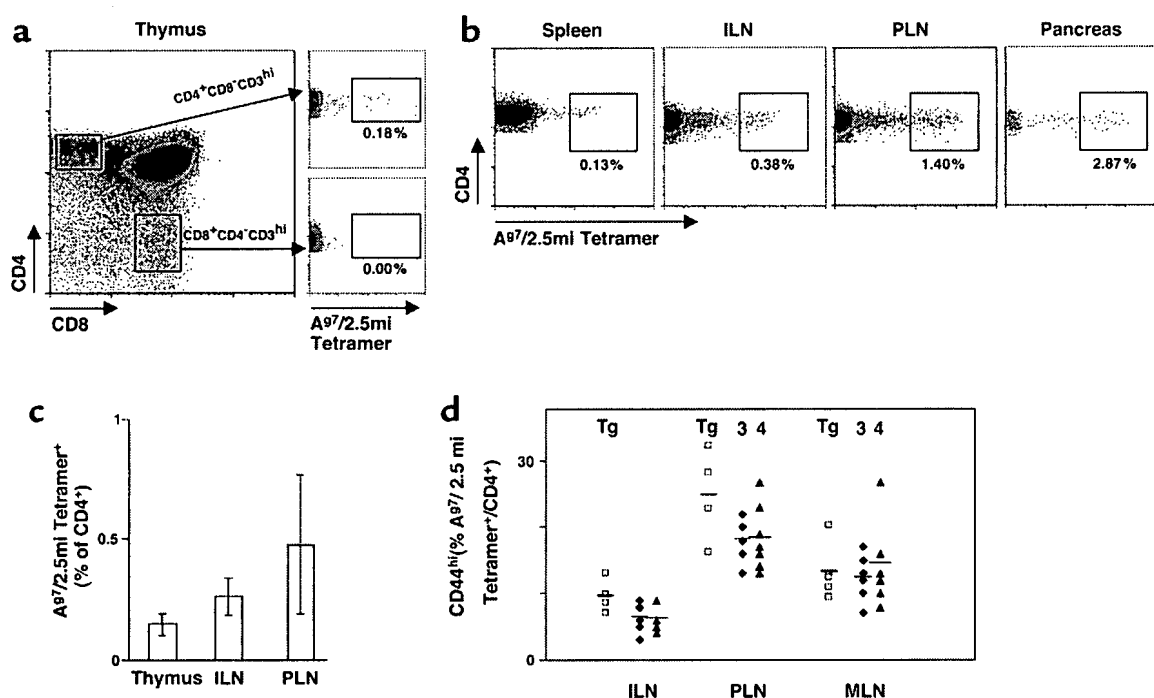
**Figure 4**  
Histological detection of 2.5mi<sup>+</sup>CD4<sup>+</sup> T cells. In situ staining of 2.5mi<sup>+</sup>CD4<sup>+</sup> T cells in PLNs (a and b) and pancreatic islets (c-e). I-A $\beta$ /2.5mi MHC tetramer stains are shown in green (a and c). Staining with an anti-CD4 antibody is shown in red and overlaid by the tetramer staining (b and d). Insulin staining is shown in e.

robust amounts of IL-2 (<200,000 counts for 95% of the clones in an NK assay, 1,500 counts for the negative control; Table 3 and data not shown). A selection of clones (with different MFIs) was further analyzed for their reactivity toward peptide and islets in the presence of APCs (Figure 7b and Tables 2 and 3). Most of the clones responded toward the peptide, though some of them with a much lower functional avidity than others ( $IC_{50}$  ranging from 0.0003 to >10  $\mu$ g/ml). We analyzed 14 clones for their reactivity to islets. Six of these clones showed reproducibly various degrees of IL-2 production (between 8% and 38% compared with BDC-2.5; Table 3 and Figure 7) as compared two five negative clones included in each experiment (a representative clone, A64, is shown in Table 3 and Figure 7). The  $V\beta$  TCR usage of the various clones, analyzed by FACS (Table 2 and Table 3), was as diverse as the staining and reactivity toward peptide, pMHC, and islet antigen.

The fact that some of these clones reacted to both peptide and islet antigen indicated strongly that the cross-reactivity between the 2.5mi peptide and a pancreatic antigen extended beyond the sole BDC-2.5 T cell to other 2.5mi-reactive T cells. These results confirmed a previous observation on the cross-reactivity of six islet-reactive, diabetogenic T cell clones with the same or similar mimotopes (41). As for the hybridomas, these clones were heterogeneous in terms of  $V\alpha/V\beta$

usage and dose-response curves. They also indicate that the anti-2.5 antigen  $CD4^+$  T cell response is diverse in terms of repertoire and range of functional avidity.

*2.5mi<sup>+</sup> T cells are present in H-2<sup>b7</sup> mice with diabetes-resistant genetic backgrounds.* What is required for the selection of 2.5mi<sup>+</sup> T cells? Do they represent defective negative selection, imparted by the diabetes-susceptibility alleles of the NOD background (15), or do they only require the presence of the A<sup>b7</sup> molecule for their selection? To test the relative contribution of MHC and background genes, we analyzed B6.H2<sup>b7</sup> and NOR, two congenic strains that carry the same H-2<sup>b7</sup> MHC haplotype as the NOD mouse but differ in other background loci. Both are fully resistant to diabetes. The 2.5mi<sup>+</sup> $CD4^+$  T cells were easily detectable in lymphoid organs of these congenic mice. Their tissue distribution (Figure 8a) and numbers (Figure 8b) were very comparable in the three H-2<sup>b7</sup> strains. Although frequencies in NOR mice tended to be slightly lower, the peripheral expansion in PLNs was always present, and, upon immunization, the expansion of 2.5mi<sup>+</sup> cells was as important as the one seen on the NOD background (Supplemental Figure 2, <http://www.jci.org/cgi/content/full/112/6/902/DC1>). Furthermore, signs of antigen-specific activation were also present in the congenic mice: the increased frequency in PLNs (Figure 8b), and the increased frequency of  $CD44^{hi}$  cells in the PLN (more pronounced in the



**Figure 5**

T cells with the 2.5mi<sup>+</sup> reactivity are positively selected in the thymus and expand in the periphery. (a and b) Thymocytes, splenocytes, inguinal LNs (ILNs), PLNs, and infiltrated pancreatic T cells were stained for CD4, CD8, CD3, and A<sup>b7</sup>/2.5mi tetramer. The percentage of A<sup>b7</sup>/2.5mi<sup>+</sup> $CD4^+$  $CD3^{hi}$  T cells was electronically calculated from the rectangle gate of each profile. (c) 2.5mi<sup>+</sup> $CD4^+$  T cells expand in the periphery after leaving the thymus, and they accumulate in the PLNs. Average values represent independent experiments for seven mice analyzed. (d) Specific activation of 2.5mi<sup>+</sup> $CD4^+$  T cells in PLNs. ILNs, PLNs, or superior mesenteric LNs (MLNs) from BDC-2.5 transgenic (Tg) mice (open squares) or from groups of 3-week-old (diamonds) or 4-week-old or older NOD mice (triangles) were costained with A<sup>b7</sup>/2.5mi tetramers and anti-CD44.

**Table 3**Characterization of selected 2.5mi<sup>+</sup> T cell hybridomas isolated from naive and peptide-immunized NOD females

Hybridoma <sup>A</sup>	Coated A87/2.5mi (ml) <sup>B</sup>	2.5mi peptide (ml) <sup>C</sup>	Islet Positive <sup>B</sup>	MFI of A87/2.5mi tetramer staining <sup>D</sup>	Vb
BDC-2.5	+++++	0.002	100%	52	4
C87	++	>1	21%	138	11
A162.7	+++++	>1	20%	25	14
C36	++++	0.3	8%	25	14
C26	+++++	0.0006	25%	25	ND <sup>E</sup>
C42	+++++	0.0003	38%	17	ND <sup>E</sup>
C1	+++++	>10	9%	56	ND <sup>E</sup>
C33	+++++	>10	12%	57	ND <sup>E</sup>
A64	0	0	0%	7	ND <sup>E</sup>

<sup>A</sup>Hybridomas isolated from naive NOD females. Hybridomas whose names begin with "A" were isolated from NOD females after 2.5mi peptide immunization. <sup>B</sup>+, 0–20%; ++, 20–40%; +++, 40–60%; +++++, 60–80%; ++++++, 80–100% of IL-2 production as compared with that of BDC-2.5 hybridoma. To test for islet reactivity, hybridomas were incubated in triplicate with irradiated APCs in the absence or presence of dissociated pancreatic islets for 24 hours in three independent experiments, and the cell supernatants were tested for IL-2 by NK cell assay. For each experiment, BDC-2.5 was used as positive control and reference. The percentage of islet reactivity relative to BDC-2.5 was calculated after subtraction of values from the non-islet control obtained from each individual hybridoma. <sup>C</sup>Concentration of 2.5mi peptide to reach 50% of activation. <sup>D</sup>MFIs for hybridomas not stained by the tetramer ranged from 2 to 2.5. <sup>E</sup>ND, not determined; hybridomas did not stain with either of the Vβ-specific antibodies used in this analysis (see Methods). The table compiles data from three independent experiments.

B6.H2<sup>g7</sup> animals) (Figure 8c). Again, the B6 mice used as a control showed no such populations. As for NOD mice, 2.5mi<sup>+</sup> T cells expanded after peptide immunization with the 2.5 mimotope in NOR mice, arguing against anergy of this cell population in a diabetes-resistant background (Supplemental Figure 2, <http://www.jci.org/cgi/content/full/112/6/902/DC1>).

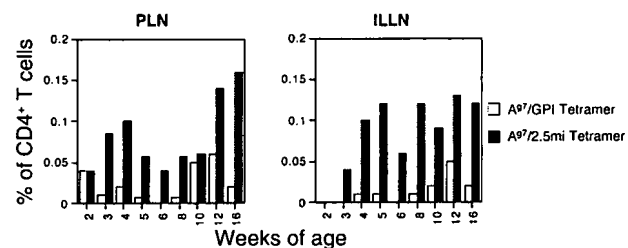
These observations allow three key conclusions: (a) MHC alleles of the H-2<sup>g7</sup> haplotype are necessary and sufficient for the selection of the 2.5mi<sup>+</sup> T cells; (b) negative selection of 2.5mi<sup>+</sup> T cells is identical in susceptible or resistant mice; and (c) 2.5mi<sup>+</sup> T cells are activated in pancreas-draining LNs regardless of the genetic background and diabetic status of the mouse.

## Discussion

The MHC multimer reagent, with its highly efficient binding to the BDC-2.5 TCR, offers a glimpse of the development of a "normal" repertoire of T cells reactive against a peripheral antigen.

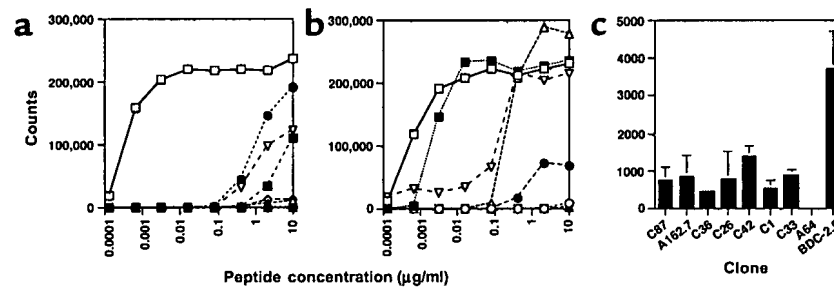
One question must be addressed at the outset. The BDC-2.5 TCR confers reactivity to a uniquely pancreatic antigen and cross-reacts with the AHHPIWARMDA mimotope analog. The 2.5mi<sup>+</sup> population of CD4<sup>+</sup> T cells binds the mimotope, but does that necessarily imply that it recognizes the original BDC-2.5 pancreatic antigen? This was not a foregone conclusion, as T cells are broadly cross-reactive but T cells of identical specificity need not share cross-reactive recognition (58). Here, the phenotype of 2.5mi<sup>+</sup> T cells in the PLN provides a decisive clue: activation of a substantial proportion of the cells is seen quite exclusively in the PLN. We and others (57, 59) have shown that activation of pancreas-specific transgenic TCRs occurs solely in the draining PLN. That a substantial proportion of the 2.5mi<sup>+</sup> T cells is also specifically activated in that LN shows these to be also reactive to a pancreas-specific antigen, and thus most likely to the BDC-2.5 antigen itself. We further confirmed this hypothesis by charac-

terizing a series of tetramer-positive T cell hybridomas. All the cells that we tested were reactive toward the 2.5 peptide, the recombinant peptide/MHC molecules, and pancreatic islets cells, confirming the cross-reactivity between the mimotope peptide and the islet antigen seen in BDC-2.5. This cross-reactivity had been previously established by characterization of mimotopes for six diabetogenic T cell clones isolated in two separate laboratories (41). All clones reacted against mimotopes retaining the central P5 tryptophan residues and common P6–P9 segments. Optimization of reactivity was achieved by changing of P2 and P3 residues. All six clones had different Vα/Vβ pairing, and three of them were activated by the mimotope that we used in the present study. The BDC-2.5 mimotope published by Judkowski et al. (40) shares the same characteristics (similar P5–P9 segment, variation at P2 and P3) and was shown to be cross-reactive with our mimotope by induction of a similar 2.5mi<sup>+</sup> cell expansion after immunization (data not shown). The relative variability of the N-terminal part of the mimotope suggests that the 2.5mi<sup>+</sup> population will be more variable in its Vα usage than in its Vβ usage. This assumption is supported by the initial TCR usage characterization that we have

**Figure 6**

Time course analysis of 2.5mi<sup>+</sup> T cells in naive NOD females. PLN and iliac LNs (ILLNs) were removed and analyzed for 2.5mi<sup>+</sup> T cells at the indicated ages. Harvested lymphocytes were pooled from four animals before analysis.





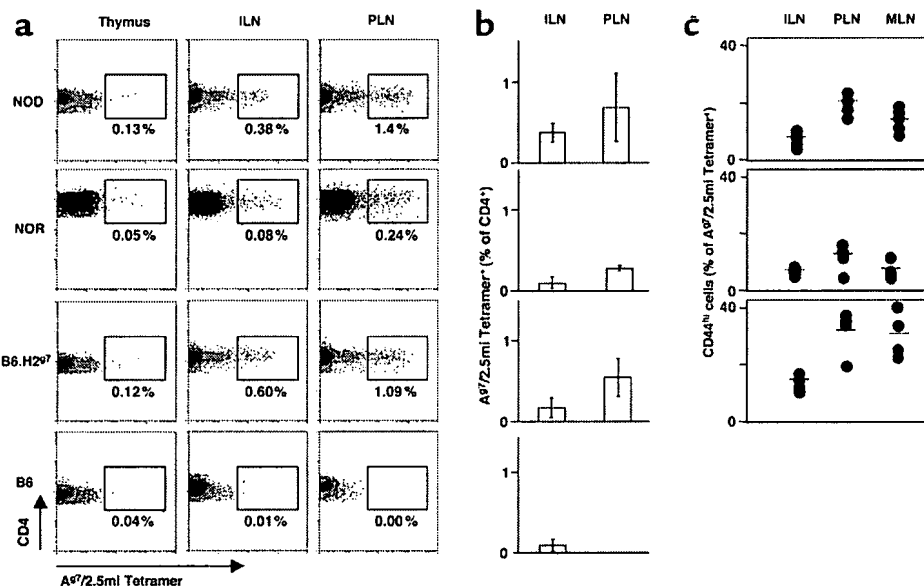
**Figure 7**

Reactivity of 2.5mi<sup>+</sup> T cell hybridomas to 2.5mi peptide and pancreatic islet cells. T cell hybridoma clones obtained from NOD females after immunization with 2.5mi peptide (a and c) or from naive animals (b and c) were incubated in the presence of APCs with increasing concentrations of 2.5mi peptide (a and b) or pancreatic islet cells (c), and their IL-2 response was measured by incorporation of <sup>3</sup>H-thymidine during an NK cell proliferation assay. Clone numbers were as follows: (a) A42, open squares; A162.7, filled circles; A202.2, filled squares; A64, open diamonds; A129, filled triangles; A129, filled diamonds; A72, open upward-pointing triangles; A86, open circles; and A166, open downward-pointing triangles; (b) C26, open squares; C20, open circles; C33, filled upward-pointing triangles; C87, filled circles; BDC-2.5, filled squares; C36, open upward-pointing triangles; C1, filled downward-pointing triangles; C26, open downward-pointing triangles.

done on the 2.5mi<sup>+</sup> population (Supplemental Figure 3, <http://www.jci.org/cgi/content/full/112/6/902/DC1>). Altogether, these data strongly suggest that BDC-2.5 and 2.5mi<sup>+</sup> T cells indeed recognize a common antigen, and that the usage of mimotope is warranted for the identification of T cell populations.

That T cells with an autoimmune specificity can exist in a healthy individual has been observed previously (43, 60). On the other hand, the very number of these 2.5mi<sup>+</sup> T cells was the first surprise. Even if more recent sequencing reports (61) have downgraded the size of the effective T cell repertoire from the 10<sup>13</sup> originally

predicted (62), the combinatorial arrangements that can make up a repertoire are such that one would not have expected nearly 0.5% of CD4<sup>+</sup> cells to bind a single MHC/peptide complex. Indeed, for other tetramer analyses, the precursor frequency in the naive repertoire was considerably lower, often not detectable but estimated to be in the range of 1 or fewer in 20,000 (27, 63–66). The frequency is already high at the outcome of thymic selection (0.18% on average) and is reflected in the naive cells of peripheral LNs (0.25% on average). In the present instance, the overrepresentation is not due to any particular Vα/Vβ combination, as the αβ



**Figure 8**

Aβ7 is sufficient to select 2.5mi<sup>+</sup>CD4<sup>+</sup> T cells. (a) Thymocytes, inguinal LNs (ILNs), and PLNs from young adult NOD mice, NOR mice, C57BL/6 mice congenic for H-2<sup>b7</sup> (B6.H2<sup>b7</sup>), and C57BL/6 (B6) mice were stained for CD4, CD8, CD3, and Aβ7/2.5mi tetramer. The percentage of Aβ7/2.5mi<sup>+</sup>CD4<sup>+</sup>CD3<sup>hi</sup> T cells was electronically calculated from the rectangle gate of each profile. (b) 2.5mi<sup>+</sup> T cells accumulate in PLNs of mice that do not get insulinitis or diabetes but share Aβ7. Average representation (percent) of 2.5mi<sup>+</sup>CD4<sup>+</sup> T cells from NOD, NOR, and B6.H2<sup>b7</sup> mice (five to seven mice per group) was measured in four independent experiments. (c) 2.5mi<sup>+</sup> T cells are activated in PLNs of mice that do not get insulinitis or diabetes but share Aβ7. The percentage of CD44<sup>hi</sup>CD4<sup>+</sup>Aβ7/2.5mi<sup>+</sup> T cells in ILNs, PLNs, and MLNs of NOD, NOR, and B6.H2<sup>b7</sup> mice (five to seven mice per group) was measured in four independent experiments.

composition of the 2.5mi<sup>+</sup> T cell repertoire is broad (T. Stratmann, unpublished observations). The overrepresentation is also reminiscent of “canonical” TCRs that dominate the response to particular antigens (67), which have been suggested to correspond to selection and maintenance on particular MHC/peptide combinations. The peculiarity, in the case of the 2.5mi<sup>+</sup> T cell population, is that this overrepresented specificity corresponds to an autoimmune clonotype. One might hypothesize that the mimotope yields a “promiscuous” surface, able to bind a variety of TCRs.

From a practical standpoint, the present observation could also mean that the prediction or early detection of diabetes based on the identification of antigen-specific T cells in at-risk patients will be difficult. If, as for the 2.5mi<sup>+</sup> specificity, frequencies are roughly the same in peripheral lymphoid organs of at-risk and healthy individuals (the human counterparts of high-risk NOD and resistant B6.H2<sup>g7</sup> mice), the sheer number of islet-reactive T cells in blood or nonpancreatic LNs may not be a good indicator of disease activity. This would be consistent with the difficulty in reproducibly distinguishing patients from controls in antigen-specific proliferation assays.

The most far-reaching conclusion that can be drawn from the present study is that the diabetes-susceptible H-2<sup>g7</sup> MHC haplotype, not the background genes, determines the selection of this autoimmune repertoire. Several lines of evidence have suggested that the genetic composition of the NOD mouse favors inefficient central tolerance, leading to the emergence of an autoimmune repertoire (14, 15). This is clearly not the case for this specificity, as the 2.5mi<sup>+</sup> T cells share similar frequencies of thymic selection and peripheral representation on the NOD, B6, or NOR background. Only H-2<sup>g7</sup> is required. On nonsusceptible B6 and NOR backgrounds, 2.5mi<sup>+</sup> T cells are fully reactive, as shown by their specific activation in the PLN (Figure 8) or by peptide-injection experiments (not shown).

But then why are NOR or B6.H2<sup>g7</sup> mice not insulinitic and diabetic? Where do background genes come into play to control diabetogenesis? Clearly, these strains can select an autoreactive repertoire of competent 2.5mi<sup>+</sup> T cells. We cannot rule out fine affinity differences in NOD versus other backgrounds, but the cells do appear similar in numbers, staining intensity, activation potential, and repertoire bias. Further, they are activated in the PLN in all strains, indicating that their presence and activation there is not pathogenic by itself. The implication is that NOD background genes are peculiar in how they allow the consequence of autoimmune selection and activation to play out after autoantigen recognition: subtle phenotypic differences imparted by APCs, which allow the activated T cells to become fully aggressive; differences in regulatory T cell populations that control the initial response; differences in tissue infiltration. That NOD background alleles

play a role by perturbing secondary immunoregulation has been proposed previously (3, 17, 43), and the notion is consistent with the fact that the recognized *idd* regions contain immunoregulatory loci: the costimulation complex on *chr1*, and the *IL-2* locus (10).

Indeed, the present observations with 2.5mi<sup>+</sup> T cells reproduce, to a striking extent, the behavior of the T cells in the original BDC-2.5 transgenic mice: robust positive selection in the thymus unencumbered by any signs of negative selection, export to the periphery in large numbers, and PLN-specific activation, all occurring with a similar independence from background genes (18, 43, 57). The present observations thus reinforce the validity of the transgenic system in analyzing immunoregulatory pathways. There is one apparent paradox: while BDC-2.5/B6.H2<sup>g7</sup> transgenic mice progress to diabetes quite readily (in fact more efficiently than mice on the NOD background) (43), B6.H2<sup>g7</sup> mice never do. If they share, at least in part, the same specificity as BDC-2.5 cells, why do 2.5mi<sup>+</sup> T cells not generate disease? The difference likely resides in the sheer numbers of clonotype-positive cells in the transgenic mouse, where allelic exclusion strongly limits the number of regulatory cells. Accordingly, the aggression of diabetes in the transgenic mouse is correlated with the number of nonclonotypic cells and is far greater if the mouse is crossed onto a RAG-deficient background (36). Here again, the difference between pathogenicity and nonpathogenicity may lie in the regulatory environment, rather than in autoimmune cells themselves.

MHC haplotypes that confer susceptibility to particular autoimmune diseases can be thought of in two different ways. First, they may favor an autoimmune repertoire by inefficient negative selection of unwanted specificities; because of poor or promiscuous binding of self-peptides imparted by the unusual structure of MHC-II molecules devoid of an Asp residue at  $\beta$ -57, specificities that are normally negatively selected would appear in the repertoire (12, 68, 69). On the other hand, one can also imagine that susceptible alleles play their role by *positively* selecting a repertoire reactive against a particular self-antigen. The binding of the similar immunodominant epitopes of islet antigens by A<sup>g7</sup> and DQ8 argues in favor of such a view (70, 71). Indeed, the DQ8 molecule can positively select the BDC-2.5 transgenic TCR (72). Here, the 2.5mi<sup>+</sup> T cells are selected in high numbers in the thymus, in a manner solely dependent on the susceptible MHC haplotype. Of course, we cannot rule out that H-2<sup>b</sup> molecules select a parallel, A<sup>b</sup>-restricted population that recognizes the same antigen. Yet it is striking that the diabetes-susceptible H-2<sup>g7</sup> haplotype can select such an abundant repertoire reactive to pancreatic self. MHC-II molecules were first identified by their capacity, as immune response genes, to present defined foreign antigens and allow the selection of a repertoire that can recognize them (73). Some alleles may selectively bring forth a repertoire targeted to self.

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# Ex Vivo Analysis of Thymic CD4 T Cells in Nonobese Diabetic Mice with Tetramers Generated from I-A<sup>g7</sup>/Class II-Associated Invariant Chain Peptide Precursors<sup>1</sup>

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The MHC determines susceptibility and resistance to type 1 diabetes in humans and nonobese diabetic (NOD) mice. To investigate how a disease-associated MHC molecule shapes the T cell repertoire in NOD mice, we generated a series of tetramers from I-A<sup>g7</sup>/class II-associated invariant chain peptide precursors by peptide exchange. No CD4 T cell populations could be identified for two glutamic acid decarboxylase 65 peptides, but tetramers with a peptide mimetic recognized by the BDC-2.5 and other islet-specific T cell clones labeled a distinct population in the thymus of young NOD mice. Tetramer-positive cells were identified in the immature CD4<sup>+</sup>CD8<sup>low</sup> population that arises during positive selection, and in larger numbers in the more mature CD4<sup>+</sup>CD8<sup>-</sup> population. Tetramer labeling was specific based on the use of multiple control tetramers, including one with a single amino acid analog peptide in which a critical TCR contact residue was substituted. The T cell population was already present in the thymus of 2-wk-old NOD mice before the typical onset of insulinitis and was detected in B10 mice congenic for the NOD MHC locus, but not B10 control mice. These results demonstrate that a T cell population can expand in the thymus of NOD mice to levels that are at least two to three orders of magnitude higher than estimated for a given specificity in the naive T cell pool. Based on these data, we propose a model in which I-A<sup>g7</sup> confers susceptibility to type 1 diabetes by biasing positive selection in the thymus and later presenting peptides from islet autoantigens to such T cells in the periphery. *The Journal of Immunology*, 2003, 171: 4175–4186.

The MHC represents a principal susceptibility locus for many autoimmune diseases, but the mechanisms by which particular MHC alleles increase the risk for a given autoimmune disease are not fully understood. The disease-associated polymorphisms map to the peptide binding site and crystallographic studies have shown that they determine the shape and charge of key pockets which accommodate peptide side chains (1). In many cases, alleles that confer susceptibility differ from non-associated alleles at only one or a few positions in the peptide binding site, implying a high degree of specificity. Peptide binding experiments have demonstrated that disease-associated MHC molecules bind peptides from candidate autoantigens, but other peptides from the same autoantigens can be bound by MHC molecules that do not confer susceptibility to the disease (2–4). The high degree of specificity implied by the genetic data could, however, be explained by a two-stage model in which the disease-associated MHC polymorphisms determine the outcome of two critical Ag presentation events: presentation of peptides in the thymus that promote positive selection of potentially pathogenic T cell populations, followed later by presentation of peptides from autoantigens to the same T cells in the target organ. This hypothesis has

been difficult to test because conventional assays of T cell function are not suitable for analysis of the thymic T cell repertoire.

Type 1 diabetes is particularly relevant for addressing this general question because a wealth of information is available on MHC genetics for the human disease as well as the nonobese diabetic (NOD)<sup>4</sup> mouse model. The MHC is the most important susceptibility locus for human type 1 diabetes and the disease-associated human HLA-DQ8 and DQ2 molecules share significant structural similarities with the I-A<sup>g7</sup> molecule expressed in NOD mice (5–10). A key polymorphic residue (β57) lacks a negative charge in both DQ8 and I-A<sup>g7</sup> and therefore does not form a salt bridge with an arginine from the α-chain (α76) (5, 6). The positively charged arginine is therefore exposed in the P9 pocket of DQ8 and I-A<sup>g7</sup>, resulting in a pocket with a preference for acidic peptide side chains. The structural similarities between DQ8 and I-A<sup>g7</sup> suggest that similar Ag presentation events are relevant in the human disease and the NOD mouse model (8–10). Other MHC class II alleles provide dominant protection from type 1 diabetes in humans and NOD mice. In humans, the DR2/DQ6 haplotype as well as several other MHC class II haplotypes significantly reduce the risk for type 1 diabetes (5, 11). In NOD mice, I-E molecules are not expressed due to a deletion in the Eα promoter and transgenic expression of the Eα gene results in protection from the disease (12–15). Protective MHC class II molecules could affect thymic selection of relevant T cell populations and/or modulate the immune response in the periphery.

Investigation of the mechanisms by which MHC molecules confer susceptibility or protection from autoimmunity thus requires analysis of CD4 T cell populations in the thymus and the target organ. Tetrameric forms of MHC class II/peptide complexes may

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<sup>4</sup> Abbreviations used in this paper: NOD, nonobese diabetic; CLIP, class II-associated invariant chain peptide; MOG, myelin oligodendrocyte glycoprotein; GAD, glutamic acid decarboxylase; HEL, hen egg lysozyme; PLP, proteolipid protein.

be useful for this purpose because labeling does not depend on a particular T cell effector function. Tetramers of murine MHC class II/peptide complexes have been generated by covalently attaching the peptide of interest to the N terminus of the MHC class II  $\beta$ -chain, but this approach requires the generation of a new transfectant/recombinant virus for every peptide of interest (16–19). Investigation of T cell populations in a spontaneous model of autoimmunity requires analysis of a panel of candidate peptides and we have developed an approach based on the cellular peptide exchange mechanism that permits a series of tetramers to be generated from a single MHC class II/class II-associated invariant chain peptide (CLIP) precursor. We initially developed this technique with human MHC class II molecules and were able to visualize virus-specific CD4 T cells in peripheral blood without prior *in vitro* culture (20). We have now examined whether this approach is applicable to the generation of tetrameric forms of murine MHC class II/peptide complexes for *ex vivo* characterization of CD4 T cells in a murine model of autoimmunity.

## Materials and Methods

### Expression and purification of I-A<sup>B7</sup>/CLIP complexes

To express soluble I-A<sup>B7</sup>, the transmembrane and cytoplasmic segments of the I- $\alpha$  and I- $\beta$  chains were replaced with leucine zipper dimerization domains from the transcription factors Fos and Jun, respectively, as previously described (21). For the purpose of the present work, several changes were made to the original constructs. To permit biotinylation of I-A<sup>B7</sup> molecules, a recognition site for the *Escherichia coli* BirA enzyme (GLNDIFEAKIEWHE, single amino acid code; Ref. 22) was attached to the C terminus of the I- $\alpha$ -Fos chain through a flexible linker (C terminus of Fos and the six amino acid linker: FILAAH-GSGSGS). The sequence representing the murine CLIP peptide was tethered to the N terminus of the mature I- $\beta$  chain through a linker with a thrombin cleavage site, permitting replacement of the CLIP peptide following purification of the protein. The protein sequence encoded by the 5' coding segment of the construct was: MALQIPSLLSAAVVVLMVLSSPGTEG (signal peptide)-GDSGT (signal peptide cleavage site and *KpnI* restriction site)-PVSQMRMAT PLLMRP (CLIP peptide)-GGGGSLLVPRGSGSGSGS (linker with thrombin cleavage site and *BamHI* restriction site)-GDSE (N terminus of mature I- $\beta$  chain). These I- $\alpha$  and I- $\beta$  constructs were cloned into the *BglII*-*EcoRI* and *BamHI* sites of the pAcDB3 vector (BD Pharmingen, San Diego, CA), respectively, under the control of the two p10 promoters located in this vector. The recombinant baculovirus that was generated with this construct was used to express the protein in High Five cells. Cells were cultured in serum-free medium (Express Five SFM; Invitrogen, Carlsbad, CA) at a density of  $1 \times 10^6$ /ml with the recombinant virus at a multiplicity of infection of 5. Supernatants were harvested 65 h following infection and the protein was purified from concentrated supernatants by affinity chromatography using mAb 10-2.16, as previously reported (21). The typical yield was ~1.8–2.5 mg/L of culture.

### Biotinylation, thrombin cleavage, and peptide exchange

Purified I-A<sup>B7</sup> molecules were biotinylated using a 1:20 molar ratio of BirA to I-A<sup>B7</sup> in a buffer containing 50  $\mu$ M biotin, 10 mM ATP, 10 mM magnesium acetate, 50 mM Bicine, and 1 $\times$  protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO) at pH 8.3. The final I-A<sup>B7</sup> concentration was adjusted to 2.0–2.5 mg/ml with 10 mM Tris (pH 8.0) and reactions were incubated for 2 hours at 30°C. Free biotin was then removed using a PD-10 size exclusion column (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated with 10 mM Tris (pH 8.0). Biotinylation was confirmed by electrophoresis on 8% native polyacrylamide gels, and dimers, trimers, and tetramers could be visualized at different molar ratios of streptavidin (Pierce, Rockford, IL) and I-A<sup>B7</sup>.

Prior to the peptide exchange reaction, the linker between the CLIP peptide and the I- $\beta$  chain was cleaved with thrombin to permit release of the CLIP peptide. Twenty units of thrombin (Novagen, Madison, WI) were used per milligram of I-A<sup>B7</sup>. The I-A<sup>B7</sup> concentration was 1–2 mg/ml and reactions were incubated at room temperature for 2 h in 10 mM Tris (pH 8.0); thrombin cleavage was confirmed by SDS-PAGE based on a shift in the molecular weight of the I- $\beta$  chain.

Peptide exchange was performed using peptides synthesized with a DNP affinity tag. The DNP group was attached during the synthesis to the N terminus of peptides via an aminohexanoic acid (Ahx) linker (Jerini, Ber-

lin, Germany); all peptides were HPLC purified and analyzed by mass spectrometry. Peptide exchange reactions were carried out with 3.3  $\mu$ M I-A<sup>B7</sup>/CLIP and 50  $\mu$ M of the respective DNP-labeled peptide in a buffer containing 50 mM sodium phosphate (pH 6.0), 100 mM NaCl, 1 mM EDTA, and 1 $\times$  protease inhibitor mixture (Sigma-Aldrich). The reactions were incubated overnight at room temperature and then concentrated by ultrafiltration. I-A<sup>B7</sup>/CLIP concentration, reaction temperature, and pH were chosen based on preliminary experiments designed to minimize aggregation of empty I-A<sup>B7</sup> molecules created by CLIP dissociation. I-A<sup>B7</sup> molecules were then separated from unbound peptide using a Superose 12 HPLC gel filtration column (Amersham Pharmacia Biotech) using PBS as running buffer at a flow rate of 0.8 ml/min. The peak representing I-A<sup>B7</sup> molecules was collected and injected onto an anti-DNP HPLC affinity column. This column was generated by covalently cross-linking 10 mg of anti-DNP-1 Ab (Biotrend Chemikalien, Cologne, Germany) to a 4.6 mm  $\times$  50 mm protein G column on POROS 20 XL medium (Applied Biosystems, Foster City, CA). I-A<sup>B7</sup> molecules with bound DNP-peptide were eluted from the column using 50 mM 3-(cyclohexylamino)-1-propane sulfonic acid (pH 11.5) and eluates were neutralized by addition of 1 M phosphate (pH 6.0). Biotinylated, peptide-loaded I-A<sup>B7</sup> molecules were concentrated by ultrafiltration (Centricon 2-ml concentrator; Millipore, Bedford, MA) and the buffer was simultaneously exchanged to 10 mM Tris (pH 8.0). Biotinylated I-A<sup>B7</sup>/peptide complexes were frozen on dry ice and stored in small aliquots at  $-80^\circ\text{C}$ .

### Labeling of T cells with MHC class II tetramers and enrichment of tetramer-positive cells with anti-PE microbeads

For tetramer formation, biotinylated I-A<sup>B7</sup>/peptide complexes were incubated with R-PE-labeled streptavidin (Molecular Probes, Eugene, OR) for at least 1 h on ice at a 4:1 molar ratio of I-A<sup>B7</sup> to streptavidin and a final I-A<sup>B7</sup> concentration of 0.1 mg/ml unless described otherwise. Four-color FACS analysis was performed with the PE-labeled tetramer and allophycocyanin-labeled anti-CD4, as well as PerCP-labeled anti-CD3 (for cells isolated from lymph nodes and spleen) or PerCP-labeled anti-CD8 (for cells isolated from thymus; all Abs were obtained from BD Pharmingen). Apoptotic cells were excluded using Alexa 488-labeled annexin V.

Magnetic enrichment of tetramer-labeled cells was used to confirm the presence of cell populations present at low frequencies. Cells were stained with tetramers (typically at 10  $\mu$ g/ml) in RPMI 1640, 10% FCS for 30 min at room temperature with gentle rocking; labeled Abs were then added and the reaction was kept on ice for another 20 min. Cells were then washed twice with PBS/1% FCS, cell pellets were resuspended in 80–100  $\mu$ l of PBS/1% FCS and 15  $\mu$ l of the anti-PE microbeads (Milenyi Biotec, Auburn, CA) were added to each sample. Following incubation for 20 min in the refrigerator, unbound beads were washed away with PBS/1% FCS and cells were resuspended in 1 ml of PBS/1% FCS. At this point, each sample was split and 900  $\mu$ l were used for magnetic enrichment while 100  $\mu$ l were kept as a nonenriched, but otherwise identically treated, sample. For enrichment, 900  $\mu$ l of the cell suspension were loaded onto the MS column (Milenyi Biotec), the column was washed three times, and cells were eluted in 2 ml of annexin-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub> (pH 7.4). Annexin V Alexa 488 conjugate (2  $\mu$ l; Molecular Probes) was added and cells were incubated for 15 min at room temperature and then placed on ice until FACS analysis. Cells that were not magnetically enriched were resuspended in 200  $\mu$ l of the diluted annexin V solution (1/1000) and treated like the magnetically enriched cells.

FACS analysis was performed using a FACSCalibur (BD Biosciences, San Diego, CA) and the CellQuest software (BD Biosciences). Lymphocytes were gated based on forward and side scatter and CD3/CD4, CD4/CD8, or CD4 expression as indicated in the figure legends.

### Enrichment of CD4 single-positive thymocytes

CD4 single-positive thymocytes were enriched from single cell suspensions by negative selection with a mixture of mAbs to CD8, CD11b (Mac-1), CD45R (B220), an erythroid marker (TER119) and a myeloid differentiation Ag (Gr-1) (SpinSep murine CD4 T cell enrichment kit; StemCell Technologies, Vancouver, British Columbia, Canada). The cell suspension (at a density of  $2-8 \times 10^7$  cells/ml) was incubated with the Ab mixture for 20 min in the refrigerator (4–10°C). Free Abs were washed away with PBS/2% FCS, and SpinSep Dense Particles (StemCell Technologies) which bind to Ab-labeled cells were then added. Following incubation for 20 min on ice, cells bound to the particles were removed by centrifugation through the SpinSep Density Medium and nonlabeled cells were recovered from the interface, washed, and resuspended in RPMI 1640, 10% FCS for tetramer staining.

### Tetramer labeling of T cells from pancreatic lymph nodes and spleen

Pancreatic lymph nodes were dissected and single cell suspensions were prepared. Cells were washed twice with PBS and then resuspended in RPMI 1640, 10% FCS for staining. For splenocytes, the single cell suspension was first washed twice with PBS and then resuspended in red cell lysis buffer (Sigma-Aldrich). Following an incubation for 10–15 min at room temperature, cells were washed twice with PBS, and resuspended in RPMI 1640, 10% FCS for staining. Magnetic enrichment of tetramer-labeled cells was performed as described above for thymocytes.

### Analysis of CD4 T cells from mice immunized with peptides

NOD mice or B10.H-2<sup>b7</sup> mice (both from Taconic Farms, Germantown, NY) were immunized s.c. with peptide emulsified in CFA (1:1 emulsion of CFA and peptide at 4 mg/ml). Popliteal lymph nodes were isolated 10 days following immunization and single cell suspensions were prepared. Cells were washed twice with PBS, resuspended in RPMI 1640 with 10% FCS, and subjected to tetramer staining. The remaining cells were cultured for 3–4 days at a density of  $5 \times 10^6$ /ml in the presence of the respective peptide (100 nM) in AIM-V medium (Invitrogen) supplemented with 2 mM L-glutamine, 55  $\mu$ M 2-ME. Viable cells were purified by Ficoll density gradient centrifugation prior to tetramer labeling.

### Peptide binding assays

Unlabeled peptides were used as competitors for binding of a biotinylated reference peptide to thrombin-cleaved I-A<sup>b7</sup>/CLIP (21). Thrombin-cleaved I-A<sup>b7</sup> molecules (200 nM) were incubated with biotinylated mouse transferrin peptide (1  $\mu$ M) in the presence of nonbiotinylated competitor peptides (40 nM to 30  $\mu$ M) overnight at 37°C in 50 mM sodium phosphate (pH 6.0), 100 mM sodium chloride, 1 mM EDTA, 100  $\mu$ g/ml BSA, and 1 $\times$  protease inhibitor mixture (Sigma-Aldrich). I-A<sup>b7</sup> bound biotinylated peptide was then quantitated with europium-labeled streptavidin following capture of I-A<sup>b7</sup> with mAb 10-2.16. For that purpose, 96-well plates (Wallac MaxiSorp F96 Low Fluorescence, Turku, Finland) were coated overnight at 4°C with 10-2.16 mAb (50  $\mu$ l/well of 4  $\mu$ g/ml) in sodium bicarbonate buffer (pH 9.6) and then washed four times with 50 mM Tris, 150 mM sodium chloride, 20  $\mu$ M EDTA, 0.05% Tween 20 to remove unbound Ab. Nonspecific binding sites were then blocked at room temperature for 2 h using 150  $\mu$ l/well assay buffer (Wallac). Plates were washed twice and the reaction samples (diluted 1/1 in assay buffer) were added to the plates (100  $\mu$ l/well). Following an incubation for 2 h at room temperature, plates were washed four times and 100  $\mu$ l of europium-labeled streptavidin (Wal-

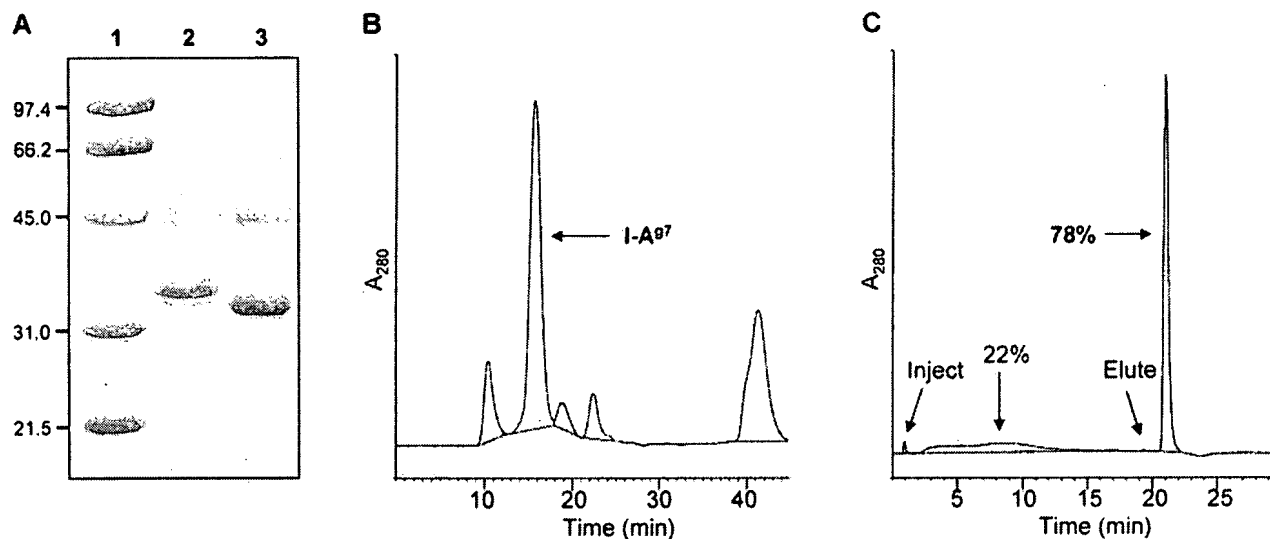
lac) was added to each well (1/2000 in 50 mM Tris, 150 mM sodium chloride, 20  $\mu$ M EDTA) for detection of the I-A<sup>b7</sup> bound biotinylated peptide. The plates were incubated for another hour and washed six times. One-hundred microliters of enhancement solution (Wallac) was then added to each well and the signal was quantitated in a fluorescence plate reader (Delfia Fluorometer; Wallac) following 30 min of incubation at room temperature.

## Results

### Generation of tetramers from I-A<sup>b7</sup>/CLIP precursors

We previously demonstrated that soluble I-A<sup>b7</sup> heterodimers could be expressed in insect cells using DNA constructs in which the transmembrane and cytoplasmic domains were replaced with leucine zipper dimerization domains from the transcription factors Fos and Jun. These soluble I-A<sup>b7</sup> molecules bound peptides known to represent I-A<sup>b7</sup> restricted T cell epitopes, and several I-A<sup>b7</sup>/peptide complexes displayed a long half-life in the absence of detergent, suggesting that tetramers generated by peptide loading would be stable. The invariant chain-derived CLIP peptide bound to I-A<sup>b7</sup> at a neutral pH but rapidly dissociated ( $t_{1/2}$  of <5 min) at pH 5.2 (21). Because “empty” MHC class II molecules have a tendency to aggregate we reasoned that the CLIP peptide could protect the hydrophobic binding site at a neutral pH during biosynthesis and be effectively exchanged with any peptide of interest at the acidic pH characteristic for the endosomal peptide-loading compartment. We expressed I-A<sup>b7</sup>/CLIP complexes as inactive precursors by attaching CLIP to the N terminus of the I-A $\beta$  chain through a linker with a thrombin cleavage site, and converted this precursor to the peptide-receptive form by linker cleavage. In APCs, the MHC class II/CLIP complex represents the natural substrate for a DM-accelerated peptide exchange reaction and is generated by proteolytic cleavage of the MHC class II bound invariant chain in the endosomal/lysosomal compartment (23–26).

I-A<sup>b7</sup>/CLIP complexes were affinity purified from the supernatant of insect cells infected with a recombinant baculovirus, with a typical yield of 1.8–2.5 mg/L of culture. Cleavage of the linker with thrombin was visualized by SDS-PAGE (Fig. 1) and peptide



**FIGURE 1.** Generation of tetramers from I-A<sup>b7</sup>/CLIP precursors. Thrombin cleavage of the linker connecting the CLIP peptide to the N terminus of the  $\beta$ -chain created the substrate for the peptide exchange reaction in which CLIP was exchanged with peptides carrying an N-terminal affinity tag. *A*, SDS-PAGE of biotinylated I-A<sup>b7</sup>/CLIP complex prior to thrombin cleavage (lane 2; 5  $\mu$ g) and purified I-A<sup>b7</sup>/peptide complex following exchange of CLIP with DNP-labeled BDC-11 peptide (lane 3; 5  $\mu$ g). Thrombin cleavage reduced the molecular weight of the  $\beta$ -chain, but did not affect migration of the  $\alpha$ -chain. *B* and *C*, Purification of I-A<sup>b7</sup>/peptide complex following the peptide exchange reaction. Following loading of I-A<sup>b7</sup> with DNP-labeled BDC-11 peptide, I-A<sup>b7</sup> molecules were separated from free peptide and aggregates by size exclusion chromatography (Superose 12; Amersham Pharmacia Biotech). The peak representing I-A<sup>b7</sup> was then injected into a HPLC DNP affinity column and bound complex was eluted by injection of 50 mM CAPS (pH 11.5). The fraction of I-A<sup>b7</sup> loaded with the DNP-labeled BDC-11 peptide was determined based on the surface area of bound vs unbound protein ( $OD_{280}$ ).

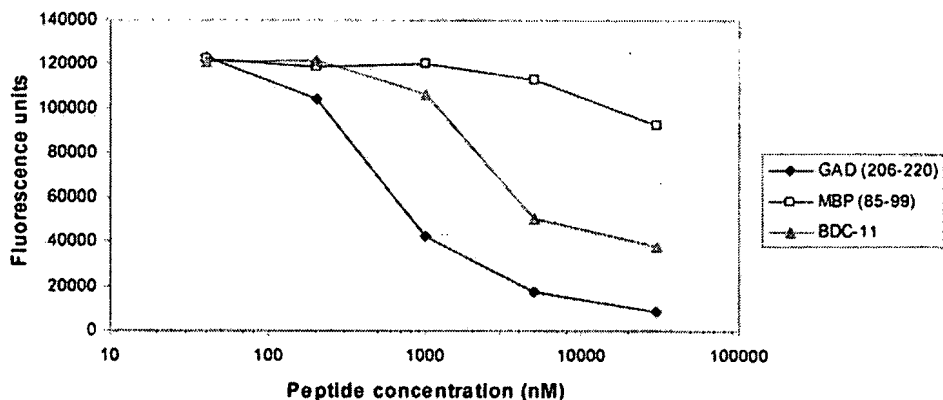
binding experiments confirmed that the CLIP peptide could be released (Fig. 2). New I-A<sup>B7</sup>/peptide complexes were generated by exchange of CLIP with peptides synthesized with an N-terminal DNP affinity tag. Two purification steps were used to isolate defined I-A<sup>B7</sup>/peptide complexes: I-A<sup>B7</sup> molecules were first separated from free peptide by HPLC gel filtration chromatography (Fig. 1B), followed by affinity purification of the complex using an anti-DNP HPLC column (Fig. 1C).

We used this peptide loading procedure to generate tetramers with peptides known to represent CD4 T cell epitopes in NOD mice. The glutamic acid decarboxylase (GAD) (206–220) peptide was identified as a major epitope for a large panel of murine T cell hybridomas that were generated from NOD mice immunized with recombinant GAD65, and we previously showed that the GAD (206–220) peptide binds with high affinity to I-A<sup>B7</sup> (3, 4). The insulin B chain (9–23) peptide was identified as the immunodominant epitope for a panel of CD4 T cell clones isolated from islets of young NOD mice (27). Several groups have also isolated islet-reactive T cell clones that recognize islet secretory Ags (28–30). Even though the molecular identity of the autoantigen(s) is not known, peptides have been identified by analysis of combinatorial peptide libraries that stimulate these clones at low peptide concentrations (28, 31). Interestingly, the majority of these clones are stimulated by the same peptide mimetic, suggesting that they recognize the same islet Ag. Several of these clones were isolated from islets of prediabetic NOD mice, while the BDC-2.5 T cell clone was isolated from the spleens and lymph nodes of newly diabetic female NOD mice. Transfer of this T cell clone induced extensive insulinitis and hyperglycemia following transfer to young NOD or NOD *scid/scid* mice, and transgenic mice that expressed the BDC-2.5 TCR developed spontaneous type 1 diabetes (32–34). You et al. (17) demonstrated that a tetramer in which one of these mimic peptides was covalently linked labeled CD4 T cells from BDC-2.5 TCR transgenic mice, as well as a small population of CD4 T cells in pancreatic lymph nodes of NOD mice.

We generated tetramers with two GAD peptides as well as several peptides representing mimics for BDC-2.5 and other islet-reactive T cell clones. The BDC-13 peptide was identical to the BDC-11 peptide, except that an alanine was added to the N and C terminus because flanking residues can stabilize the MHC/peptide/TCR interaction (35). In the BDC-11 W→R peptide, a critical

TCR contact residue of the BDC-11 peptide was changed from tryptophan to arginine. The BDC-Osa tetramer was generated with a different mimic peptide that also stimulated the BDC-2.5 and other islet-specific T cell clones. In addition, we generated several control tetramers in order to probe specificity of tetramer labeling. These controls included peptides from OVA (residues 323–339), HIV p24 (p24, residues 33–46) and myelin oligodendrocyte glycoprotein (MOG, residues 1–20) (Table I); the MOG and HIV peptides were identified using peptide binding experiments, while the OVA peptide was previously shown to bind to I-A<sup>B7</sup> (36). Tetramers could be generated with all peptides that had an IC<sub>50</sub> of <30  $\mu$ M in the competition assay (Fig. 2), indicating that peptides with a wide range of affinities were suitable. The insulin B chain (9–23) peptide had a very low affinity for I-A<sup>B7</sup> (IC<sub>50</sub> of >30  $\mu$ M) and rapidly dissociated from I-A<sup>B7</sup>. The complex could be generated by performing the purification steps at 4°C, but was not stable; generation of this tetramer by peptide loading would therefore require modification of peptide anchor residue(s) that increase the affinity of binding to I-A<sup>B7</sup>. For all other peptides, efficient peptide loading was observed because >50% of I-A<sup>B7</sup> molecules were occupied with DNP-labeled peptides, based on the surface area of the peaks in the DNP affinity purification step representing bound and unbound protein, respectively (Fig. 1C, Table I). The final protein yields were excellent considering that two purification steps were performed following the peptide loading reaction (Table I, Fig. 1).

The functionality of tetramers generated by this peptide exchange procedure was assessed by immunization of NOD mice with the GAD (206–220) or BDC-11 peptide in CFA and labeling of short-term T cell lines generated from draining lymph nodes with the respective tetramers (the peptides used for immunization did not carry the DNP group). The tetramer with the GAD (206–220) peptide labeled only CD4 T cells from mice immunized with the GAD peptide, but not from BDC-11 immunized mice (Fig. 3). Conversely, the tetramer with the BDC-11 peptide labeled CD4 T cells from mice immunized with the BDC-11 peptide, but not from GAD (206–220) immunized mice. In addition, cells from neither T cell line were labeled by the CLIP control tetramer. These results demonstrated that the peptide exchange procedure was suitable for the generation of tetramers of I-A<sup>B7</sup>/peptide complexes.



**FIGURE 2.** Peptide binding to I-A<sup>B7</sup>/CLIP precursors. A competition assay was used to compare the binding of GAD (206–220) and BDC-11 peptides to I-A<sup>B7</sup>/CLIP, with the myelin basic protein (MBP) (85–99) peptide serving as a negative control. Thrombin-cleaved I-A<sup>B7</sup> molecules (200 nM) were incubated with biotinylated mouse transferrin peptide (1  $\mu$ M) in the presence of nonbiotinylated competitor peptides (40 nM to 30  $\mu$ M) overnight at 37°C. I-A<sup>B7</sup>-bound biotinylated peptide was then detected with europium-labeled streptavidin following capture of I-A<sup>B7</sup> with mAb 10-2.16. Even though the affinity of the BDC-11 peptide for I-A<sup>B7</sup> was lower than for GAD (206–220), tetramers could be generated with both peptides based on the exchange procedure.



Table I. *I-A<sup>b7</sup>* Tetramers used for analysis of CD4 T cells in NOD mice<sup>a</sup>

Peptide	Sequence	Loading Efficiency (%)	Final Yield (μg)
CLIP	PVSQMRMATPLLMRP	NA	490
OVA (323–339)	ISQAVHAHAHAEINEAGR	58	230
HIV p24 (33–46)	SPEVIPMFSAISEG	63	240
MOG (1–20)	GQFRVIGPRHPRALVGDEV	72	180
BDC-11	AVRPLWVRMEA	78	230
BDC-11 W→R	AVRPLRVRMEA	58	210
BDC-13	AAVRPLWVRMEA	78	240
BDC-15	AAAVRPLWVRMEA	66	280
BDC-Osa	AAHHPIWARMDA	81	290
GAD (206–220)	TYETAPVFVLLLEYVT	ND	460
GAD (471–490)	VDKCLEAEYLYNIKNREG	ND	180

<sup>a</sup> A series of tetramers was generated for the analysis of CD4 T cell populations in NOD mice, with the first four (CLIP, OVA 323–339, HIV p24, and MOG 1–20) representing controls. The loading efficiency represents the fraction of *I-A<sup>b7</sup>* protein injected into the DNP affinity column that was loaded with DNP-labeled peptide, based on the surface area of the eluted peak and the flowthrough ((area of eluted peak/area of flowthrough peak + area of eluted peak) × 100). The final yield represents the amount of *I-A<sup>b7</sup>*/peptide complexes following peptide loading and purification, starting from 1 mg of biotinylated *I-A<sup>b7</sup>*/CLIP. All synthetic peptides carried an N-terminal DNP group, with the exception of the two GAD peptides. The CLIP peptide represents the sequence encoded by DNA sequence in the expression construct. NA, not applicable; ND, not determined.

### *Ex vivo analysis of T cell populations in NOD mice by magnetic enrichment of tetramer-labeled cells*

We next determined whether such tetramers were suitable for *ex vivo* analysis of murine CD4 T cell populations. To examine these cell populations independent of the autoimmune process in NOD mice, we first immunized B10.H-2<sup>b7</sup> mice with the GAD (206–220) or BDC-11 peptides and labeled cells from draining lymph nodes 10 days following immunization, without prior *in vitro* culture (Fig. 4). The GAD (206–220) tetramer labeled a substantial population of CD4 T cells (0.65%) from lymph nodes of GAD (206–220) immunized mice, while background labeling with the CLIP tetramer was low (0.01%). The tetramer-positive population was highly enriched by use of anti-PE microbeads prior to FACS analysis (18.89% of CD4 T cells). A large population was present in draining lymph nodes of mice immunized with the BDC-11 peptide (1.78% tetramer-positive cells), and a striking enrichment to 77.65% of CD4 T cells was observed following isolation with anti-PE microbeads. The BDC-13 tetramer was used because it typically provided brighter staining than the BDC-11 tetramer; increasing peptide length to a 15 mer (BDC-15) did not result in a further increase in the intensity of staining (data not shown). Magnetic enrichment thus resulted in a clear delineation of tetramer-

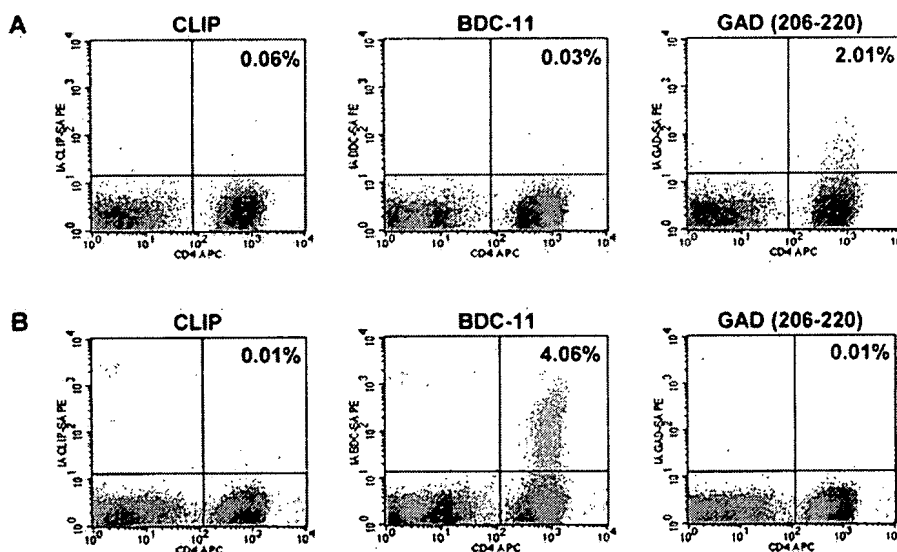
positive and -negative populations and enhanced detection of brightly labeled cells that may express TCRs with relatively high avidities for the respective *I-A<sup>b7</sup>*/peptide complex.

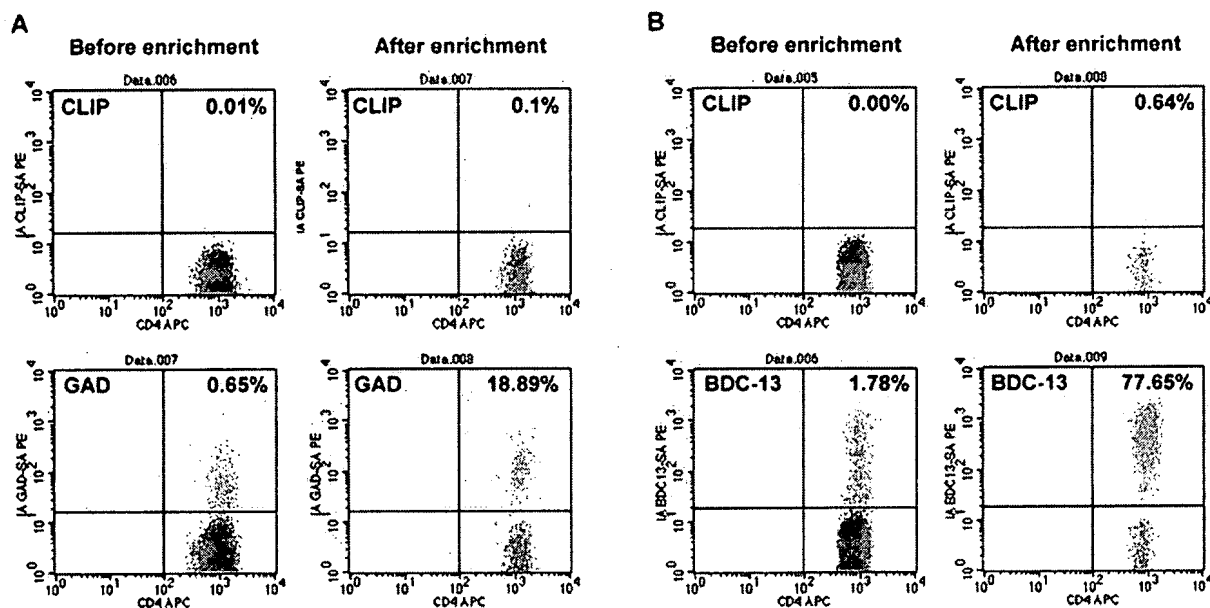
Analysis of pancreatic lymph nodes from young, nonimmunized NOD mice demonstrated that the enrichment procedure was also suitable for the detection of naturally expanded T cell populations (Fig. 5). T cells that bound the BDC-13 tetramer were detected at a frequency of 0.07%, which was substantially higher than for the control tetramers. Magnetic enrichment of cells labeled with the BDC-13 tetramer yielded a distinct, brightly labeled population (5.1% of CD4 T cells), while no discrete population was identified with the three control tetramers.

### *Detection of T cells labeled with the BDC tetramer in the thymus of young NOD mice*

In newborn mice, very few T cells can be recovered from peripheral lymphoid structures. Only  $0.14 \times 10^6$  CD4 T cells were isolated from the spleen of 2-day-old mice, compared to  $16.75 \times 10^6$  from 6- to 8-wk-old C57BL/6 mice (37). Peripheral T cell numbers thus increase >100-fold during the first weeks of life and the initial development of insulinitis at 3–4 wk of age in NOD mice may

**FIGURE 3.** Functionality of tetramers generated by peptide exchange. NOD mice were immunized s.c. with either GAD (206–220) or BDC-11 peptides in CFA. Cells from popliteal lymph nodes were cultured for 3 (BDC-11) or 4 days (GAD 206–220) in serum-free medium with the immunizing peptide at 100 nM (peptides used for immunization and culture were not DNP-labeled). Following isolation of viable cells by Ficoll density gradient centrifugation, cells were labeled with tetramers (20 μg/ml) for 2 h at 37°C, and PerCP-labeled CD3 and allophycocyanin-labeled CD4 Abs were added during the last 30 min of incubation. Cells were then stained with Alexa 488 annexin V to permit exclusion of apoptotic cells and gated based on forward light and side light scatter, annexin V, and anti-CD3 labeling. Histograms show staining with PE-labeled tetramers vs allophycocyanin-labeled anti-CD4.

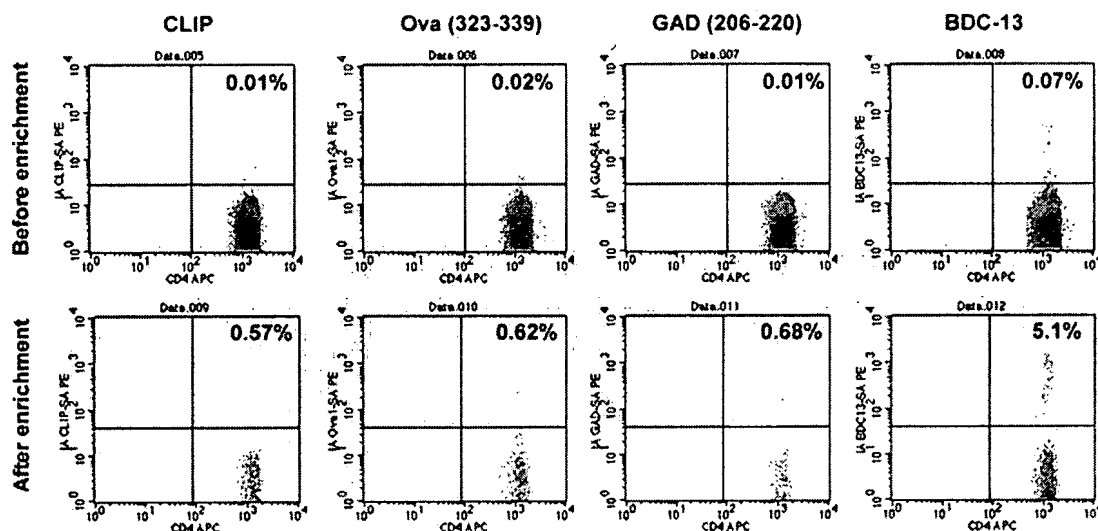




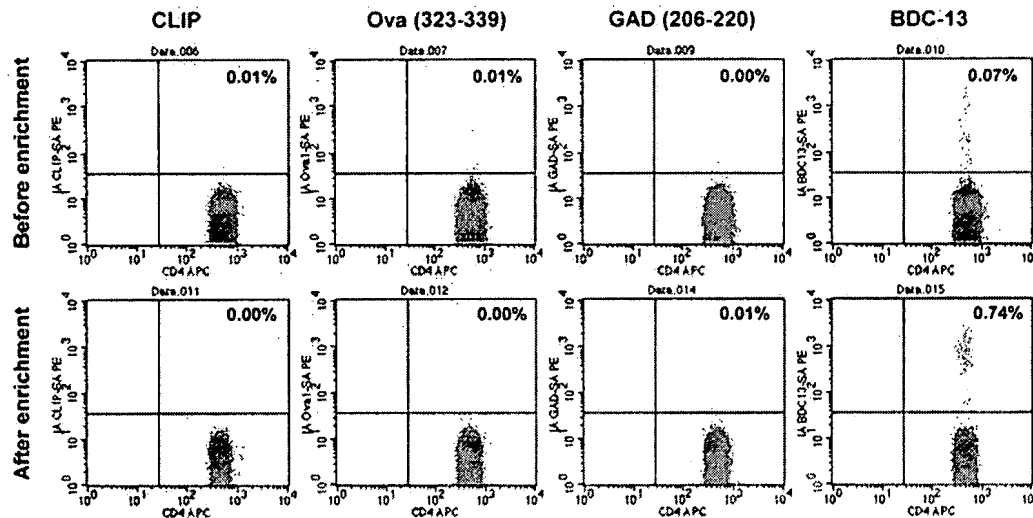
**FIGURE 4.** Enrichment of T cells labeled ex vivo with tetramers. B10.H-2<sup>b7</sup> congenic mice were immunized with the GAD (206–220) or BDC-11 peptides in CFA and cell suspensions from popliteal lymph nodes were labeled with tetramers without prior in vitro culture. To minimize tetramer internalization, tetramer labeling (20  $\mu$ g/ml for A; 10  $\mu$ g/ml for B) was performed at room temperature for 30 min, followed by incubation on ice for 20 min after addition of PerCP-labeled CD3 and allophycocyanin-labeled CD4 Abs. Cells were then washed and labeled with anti-PE microbeads. A total of  $5 \times 10^6$  cells were used per staining reaction and 90% of these cells were used for magnetic enrichment while the remaining 10% of cells were not enriched following labeling with anti-PE microbeads. Tetramer-PE vs CD4-allophycocyanin staining was plotted for the annexin V-negative, CD3<sup>+</sup>CD4<sup>+</sup> population.

thus coincide with seeding of the periphery by thymic emigrants. Therefore, we examined whether the initial expansion of T cells labeled with the BDC-13 tetramer had occurred in the thymus (Fig. 6). Because the majority of thymocytes represent CD4<sup>+</sup>CD8<sup>+</sup> T cells, we enriched CD4<sup>+</sup>CD8<sup>−</sup> cells by negative selection using a mixture of Abs to CD8 (depletion of CD8 single-positive and double-positive cells), B220, CD11b, and other markers. This enrichment procedure reduced background staining and also permitted analysis of larger numbers of CD4<sup>+</sup>CD8<sup>−</sup> cells. A distinct population of tetramer-positive CD4<sup>+</sup>CD8<sup>−</sup> T cells was only identified

with the BDC-13 tetramer, but none of the control tetramers. Following enrichment, a brightly labeled discrete cell population was observed for the BDC-13 tetramer (0.74% of CD4 T cells), but none of the control tetramers (0.01 or less of CD4 T cells). Therefore, the enrichment procedure provided a clear distinction between the BDC-13 tetramer and the control tetramer, and also permitted clear separation of tetramer-positive and -negative cells. No T cell populations were detected in the thymus of NOD mice with the GAD (471–490) tetramer or the MOG (1–20) control tetramer (data not shown).



**FIGURE 5.** Ex vivo analysis of T cells in pancreatic lymph nodes. Cells from pancreatic lymph nodes of 4-wk-old NOD mice (30 days old) were stained with PE-labeled CLIP, OVA (323–339), GAD (206–220), and BDC-13 tetramers. Cells were first stained with tetramers (10  $\mu$ g/ml) for 30 min at room temperature, followed by incubation on ice for 20 min after addition of an allophycocyanin-labeled CD4 Ab. A total of  $3 \times 10^6$  cells were used per staining reaction and 90% of the cells were used for magnetic enrichment (lower row) while the remaining 10% were not enriched (upper row) following labeling with anti-PE microbeads. Tetramer-PE vs CD4-allophycocyanin staining was plotted for the annexin V-negative, CD4-positive population.

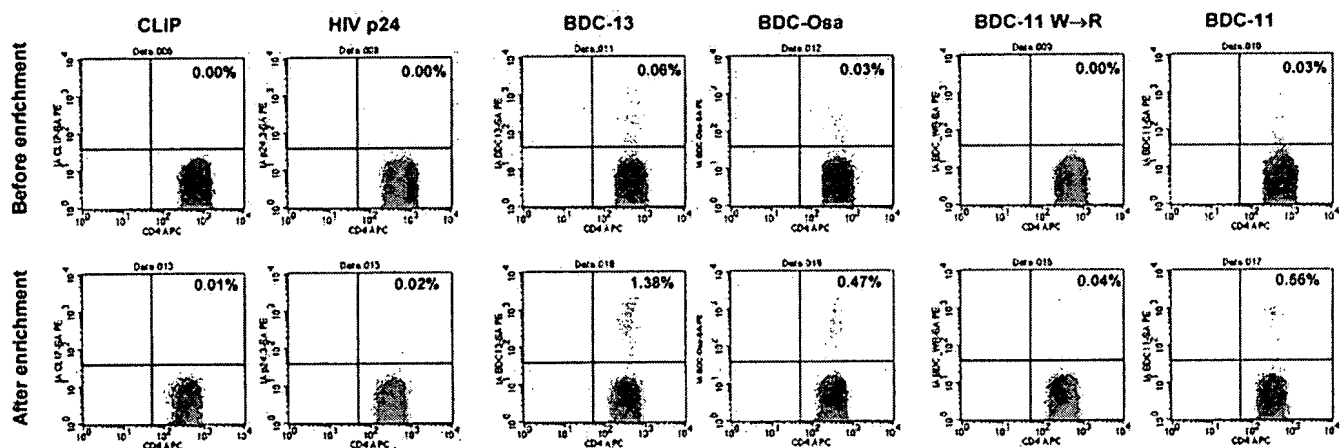


**FIGURE 6.** Ex vivo detection of T cells in the thymus of 4-wk-old NOD mice.  $CD4^+CD8^-$  thymocytes from 4-wk-old (28 days old) NOD mice were enriched by negative selection with a mixture of mAbs to CD8, CD11b, CD45R, an erythroid marker (TER119), and a myeloid differentiation Ag (Gr-1). Cells were first stained with tetramers ( $10 \mu\text{g/ml}$ ) for 30 min at room temperature, followed by incubation on ice for 20 min after addition of PerCP-labeled CD8 and allophycocyanin-labeled CD4 Abs. A total of  $4 \times 10^6$  cells were used per staining reaction and 90% of the cells were used for magnetic enrichment (lower row) while the remaining 10% were not enriched (upper row) following labeling with anti-PE microbeads. Tetramer-PE vs CD4-allophycocyanin staining was plotted for the annexin V-negative,  $CD4^+CD8^-$  population.

Because the molecular identity of the islet Ag recognized by BDC T cells is not known, a critical question relates to the specificity of tetramer labeling. We have used two approaches to address this question (Fig. 7). The first is based on the fact that a number of different peptide mimetics have been identified that activate T cells specific for this islet Ag (28, 31). For two of these peptides, only four critical residues in the nine amino acid core are identical but both peptides nevertheless stimulate BDC-2.5 T cells. Tetramers with both mimic peptides (BDC-13 and BDC-Osa) labeled a discrete population of CD4 T cells in the thymus of young NOD mice, showing that the TCR specificity of these cells corresponded to that reported for BDC-2.5 and other T cell clones. As a second approach, we generated a tetramer with a single amino acid analog peptide in which a critical TCR contact residue was substituted. Analysis of T cell clones and hybridomas demonstrated that the tryptophan present at position 5 (relative to the P1

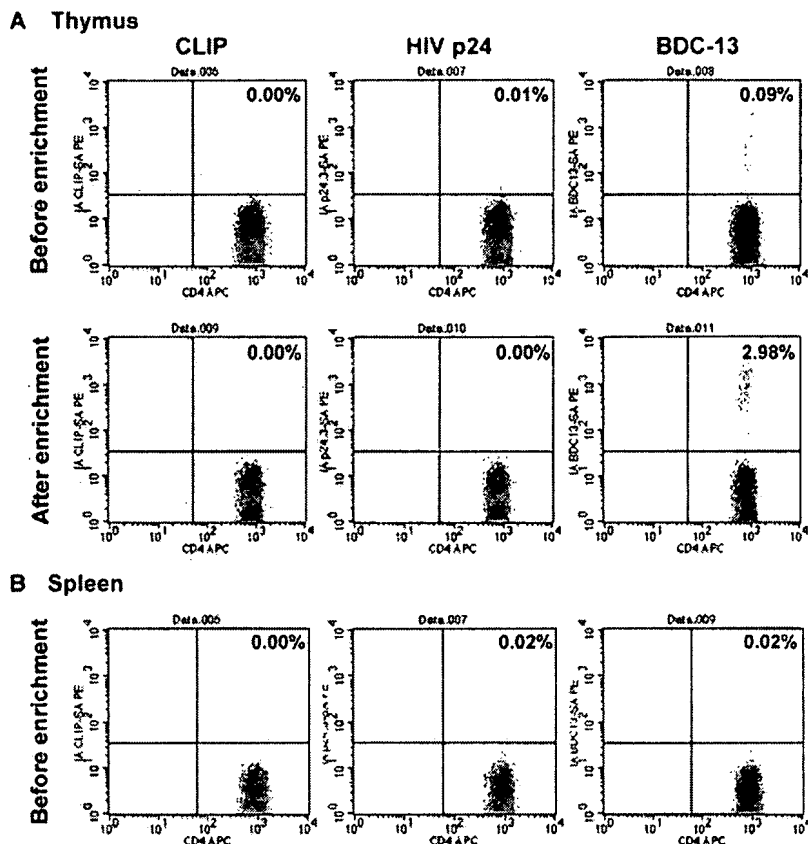
anchor residue) was critical and that a number of substitutions, including a change to arginine, abrogated T cell activation (28, 31). Therefore, we generated a tetramer with this single amino acid substitution (BDC-11 W $\rightarrow$ R) and found that this substitution greatly reduced the frequency of T cells that were detected compared to the BDC-11 tetramer (from 0.56 to 0.04% in samples enriched with anti-PE beads). Among this set of tetramers, the BDC-13 tetramer gave the brightest staining and labeled the largest cell population, while the staining intensity of the BDC-Osa tetramer was similar to BDC-11. These experiments thus demonstrate a high degree of specificity of tetramer labeling.

In 4-wk-old mice, T cells labeled with the BDC-13 tetramer could already be detected in both thymus and spleen. To define the site of initial expansion, we compared thymic and splenic cell populations in 15-day-old NOD mice (Fig. 8). T cells labeled by the BDC-13 tetramer could only be detected in the thymus but not in



**FIGURE 7.** Specificity of tetramer labeling of thymocytes.  $CD4^+CD8^-$  thymocytes were enriched from the thymus of 4.5-wk-old NOD mice (32 days old) as described as in Fig. 6 and labeled with six different tetramers: two negative control tetramers (CLIP and HIV p24), two tetramers with mimic peptides that both represent agonists for BDC-2.5 T cells (BDC-13 and BDC-Osa), and two tetramers with 11-mer peptides (BDC-11 and BDC-11 W $\rightarrow$ R) in which one of the peptides carried a substitution (W $\rightarrow$ R) at a critical TCR contact residue. A total of  $4 \times 10^6$  cells were used per staining reaction and 90% of the cells were used for magnetic enrichment (lower row) while the remaining 10% were not enriched (upper row) following labeling with anti-PE microbeads. Tetramer-PE vs CD4-allophycocyanin staining was plotted for the annexin V-negative,  $CD4^+CD8^-$  population.

**FIGURE 8.** T cells labeled by BDC tetramer are first detected in the thymus. Thymocytes (enriched for  $CD4^+CD8^-$  cells, *A*) and splenocytes (*B*) from 15-day-old NOD mice were analyzed. Cells were first stained with tetramers ( $10 \mu\text{g/ml}$ ) for 30 min at room temperature, followed by incubation on ice for 20 min after addition of allophycocyanin-labeled CD4 as well as PerCP-labeled CD8 (thymocytes) or PerCP-labeled CD3 (splenocytes) Abs. A total of  $5 \times 10^6$  cells were used per staining reaction and 90% of the cells were used for magnetic enrichment while the remaining 10% were not enriched following labeling with anti-PE microbeads. A population of T cells labeled with the BDC-13 tetramer was only detected in the thymus, but not in the spleen at this age.



the spleen at this age. In the thymus, the following numbers of tetramer $^+$ /CD4 $^+$  cells were detected following enrichment: CLIP and HIV p24 control tetramers, 0 cells; BDC-13 tetramer, 133 cells. Following enrichment, the following numbers of tetramer $^+$ /CD4 $^+$  cells were detected among splenocytes: CLIP and HIV p24 control tetramers, 0 cells; BDC-13 tetramer, 1 cell (data not shown). This result demonstrated that the T cell population detected with the BDC tetramers originated in the thymus and had not recirculated from secondary lymphoid organs.

Positive selection occurs during the transition from CD4 $^+$ CD8 $^+$  double-positive to single-positive thymocytes (38) and we therefore examined at which point T cells could be detected with the BDC-13 tetramer. Suspensions of nonfractionated thymocytes were labeled with CLIP or BDC-13 tetramers and four populations were defined based on CD4 and CD8 expression, with P1 corresponding to CD4 $^+$ CD8 $^-$  and P2 to CD4 $^+$ CD8 $^{\text{low}}$  cells (Fig. 9). T cells labeled with the BDC-13 tetramer could not be identified in the double-positive population (P3) or CD8 $^+$ CD4 $^-$  single-positive cells (P4). However, BDC-13 tetramer-positive cells were present in the intermediate CD4 $^+$ CD8 $^{\text{low}}$  population (P2) and a significant enrichment was observed with anti-PE microbeads (from 0.01 to 0.25%; 25-fold enrichment). Substantially larger numbers were present in the more mature CD4 $^+$ CD8 $^-$  population (0.08%, enriched to 9.47%; >100-fold enrichment).

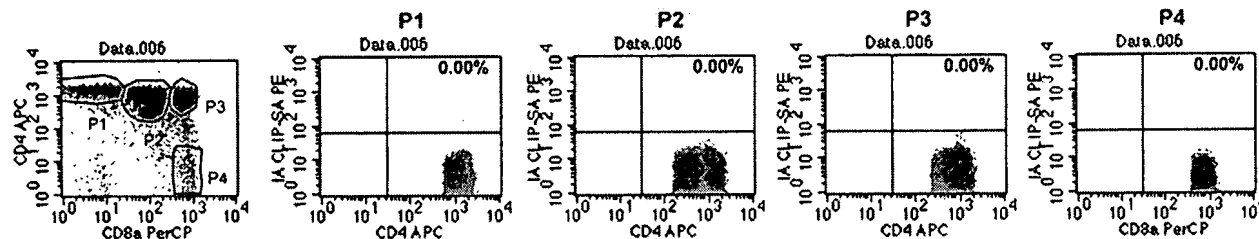
We also examined tetramer labeling for thymocytes from B10 control mice (H-2 $^b$  haplotype) and B10 mice congenic for the MHC locus of NOD mice (B10.H-2 $^g$ ) (Fig. 10). The BDC-13 tetramer did not identify a cell population in the thymus of B10 mice, but labeled a distinct population in the thymus of B10.H-2 $^g$  mice. These results demonstrate that the presence of the MHC locus of NOD mice on a different genetic background is sufficient for positive selection of this T cell population in the thymus.

## Discussion

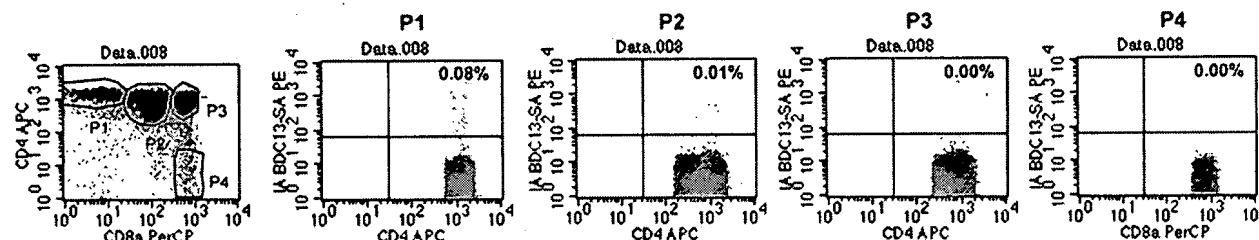
These results demonstrate that positive selection in the thymus of NOD mice creates substantial numbers of CD4 $^+$ CD8 $^-$  T cells that bind I-A $^g$ /BDC tetramers. The frequency of these cells is at least two to three orders of magnitude higher than the average precursor frequency estimated for T cells with a given MHC/peptide specificity in the naive T cell pool ( $1:10^5$  to  $1:10^6$  or lower). An expanded population of these T cells was also observed in the thymus of B10 mice congenic for H-2 $^g$ , indicating that the NOD MHC genes were sufficient on a different genetic background. Tetramer labeling was specific, based on a number of criteria: 1) discrete cell populations were not detected in the thymus of NOD mice with a panel of control tetramers; 2) the tetramer-labeled cell population could be significantly enriched with anti-PE microbeads, while no enrichment of cells labeled with control tetramers was observed; 3) the cell population was present in the thymus of NOD and B10.H-2 $^g$ , but not B10 control mice; 4) staining was greatly reduced by a single amino acid substitution in the peptide known to affect activation of T cell clones/hybridomas reactive with the islet autoantigen; 5) two mimic peptides known to stimulate the same islet-specific T cell clones labeled this thymic T cell population, even though these peptides only shared sequence identity at four positions within the nine amino acid core. Even though activated T cells can recirculate into the thymus (39, 40), T cells detected with the I-A $^g$ /BDC tetramers had expanded in the thymus due to efficient positive selection and did not represent a recirculating population because they could be detected in the thymus, but not the spleen, of 2-wk-old NOD mice (Fig. 8). You et al. (17) previously reported detection of a CD4 T cell population labeled with an I-A $^g$ /BDC tetramer in pancreatic lymph nodes and the spleen of NOD mice. They demonstrated that these cells proliferated in

A

## a CLIP control tetramer – before enrichment

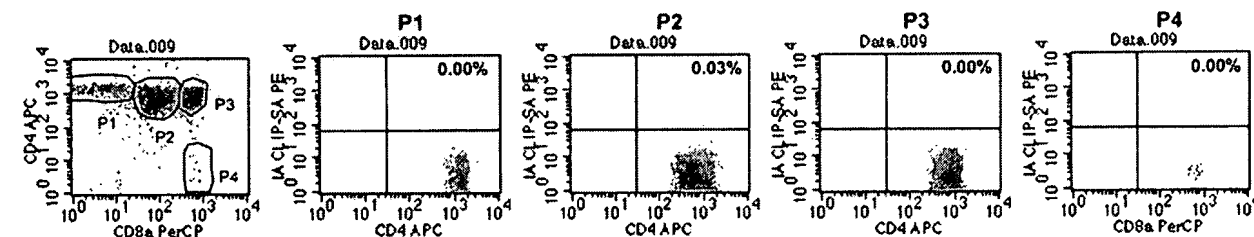


## b BDC-13 tetramer – before enrichment

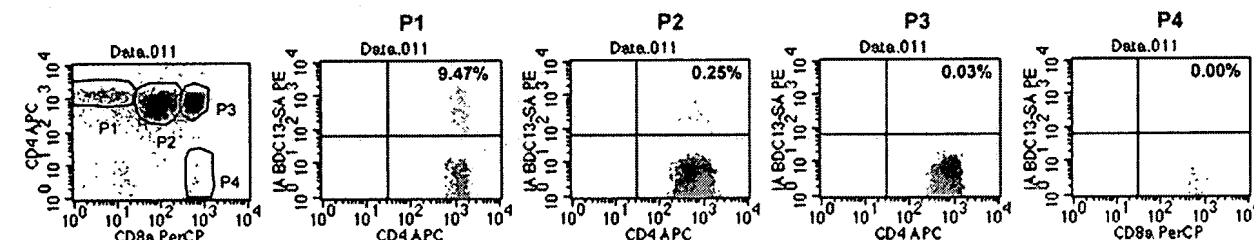


B

## a CLIP control tetramer – after enrichment



## b BDC-13 tetramer – after enrichment

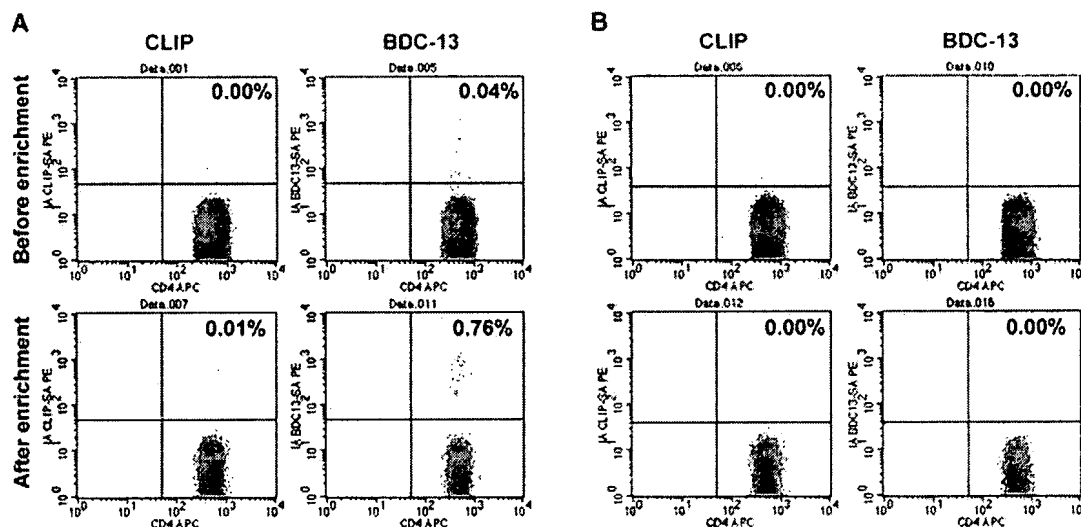


**FIGURE 9.** Analysis of T cell subpopulations in the thymus of NOD mice. Total thymocyte cell suspensions from 40-day-old NOD mice were labeled with CLIP or BDC-13 tetramers as well as PerCP-labeled CD8 and allophycocyanin-labeled CD4 Abs. Because a large fraction of thymocytes were  $CD4^+CD8^+$ , a total of  $5 \times 10^7$  cells were used per staining reaction to permit adequate analysis of the other subpopulations. Ninety percent of the cells were used for magnetic enrichment (*B*) while the remaining 10% were not enriched (*A*) following labeling with anti-PE microbeads. For the nonenriched population, only  $1 \times 10^6$  labeled cells were analyzed. Based on CD4 and CD8 labeling, four populations (P1–P4) were defined and tetramer-PE vs CD4-allophycocyanin staining plotted for annexin V-negative cells.

vitro and produced IFN- $\gamma$  and other cytokines, indicating that the cells were functional (17).

Two groups have isolated a series of T cell clones reactive with an islet secretory granule Ag, with the T cells originating from islets of prediabetic NOD mice or spleen/lymph nodes of diabetic NOD mice (28, 30). These clones cause diabetes following transfer to NOD *scid/scid* mice, and the BDC-2.5 TCR has been used to generate TCR transgenic mice that develop spontaneous diabetes (32–34). The native autoantigen is not known, but analysis of combinatorial peptide libraries has provided a series of peptide mimetics that stimulate these T cell clones/hybridomas at low peptide concentrations (28, 31). Surprisingly, six of seven independent

clones/hybridomas were stimulated by the same peptide mimetics, indicating that the majority of these clones have the same Ag specificity. This analysis also delineated key structural features of the peptides required for T cell activation. Within the nine amino acid core segment of these peptide mimetics, four positions were particularly important (P3, P5, P7, P8) while a number of substitutions were tolerated at the other five positions. The BDC-2.5 T cell clone was stimulated by two peptides in which only these four positions were conserved (P-W-R-M at P3, P5, P7, and P8) while the other positions were distinct (VR-L-V-E and HI-I-A-D at P1, P2, P4, P6 and P9). The substitutions at three of these five positions were relatively conservative (leucine/isoleucine at P4, valine/alanine at



**FIGURE 10.** H-2<sup>87</sup> haplotype is sufficient for positive selection of CD4 T cells identified with BDC tetramer. CD4<sup>+</sup>CD8<sup>-</sup> thymocytes from B10 mice congenic for the NOD MHC locus (B10.H-2<sup>87</sup>) and B10 control mice were enriched by negative selection as described in Fig. 6 and labeled with four different tetramers (CLIP, HIV p24, GAD 206–220, BDC-13; data are shown for CLIP and BDC-13 tetramers). Cells were first stained with tetramers (10  $\mu$ g/ml) for 30 min at room temperature, followed by incubation on ice for 20 min after addition of PerCP-labeled CD8 and allophycocyanin-labeled CD4 Abs. A total of  $5 \times 10^6$  cells were used per staining reaction and 90% of the cells were used for magnetic enrichment (lower row) while the remaining 10% were not enriched (upper row) following labeling with anti-PE microbeads. Tetramer-PE vs CD4-allophycocyanin staining was plotted for the annexin V-negative, CD4<sup>+</sup>CD8<sup>-</sup> population. No staining was observed with HIV p24 or GAD (206–220) tetramers in B10 or B10.H-2<sup>87</sup> mice (data not shown).

P6, glutamic acid/aspartic acid at P9). The divergent positions are largely buried in the I-A<sup>87</sup> binding groove (P1, P4, P6, and P9 pockets), and previous studies on other peptide mimetics have demonstrated that the MHC binding surface can be diverse (41). I-A<sup>87</sup> tetramers with both mimetics labeled the CD4 T cell population in the thymus of NOD mice, indicating that the structural requirements for TCR recognition are similar for the thymic T cell population studied here and the T cell clones isolated by other research groups. Molecular characterization of the native target Ag will be required for tetramer-based analysis of this T cell population with the native peptide.

The identification of autoreactive CD4 T cells with tetramers of MHC class II/peptide complexes has represented a significant challenge, due to technical difficulties in the expression of MHC class II/peptide complexes and the relatively low frequency of autoreactive CD4 T cells. In addition, the avidity of TCR for self-peptide/MHC complexes may in general be lower than those for microbial peptide/MHC complexes due to thymic and peripheral tolerance mechanisms (42). Peptide mimetics may be useful for the detection of autoreactive CD4 T cells because peptides with the highest biological activity can be chosen from a large number of variants. This concept was elegantly illustrated by the use of the NRP-V7 peptide mimetic recognized by an islet-specific CD8 T cell population. A tetramer with this peptide could be used to predict the development of type 1 diabetes in NOD mice based on labeling of CD8 T cells from peripheral blood (43). T cells labeled with this tetramer were also detected in islets, starting at 7 wk of age and peaking at 11–14 wk, at which time the frequency of these cells ranged from 1.61 to 36.8% of CD8 T cells in individual mice. The brightness of tetramer labeling and consequently the size of the detected cell population had been optimized based on single amino acid substitutions at position 7 of the peptide (W, A, or V) and the population labeled with the NRP-V7 tetramer was 4- to 5-fold larger than the one stained with the NRP-A7 tetramer. Recent work has demonstrated that the self-peptide recognized by these CD8 T cells is derived from the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) and that tet-

ramers loaded with the IGRP peptide label a CD8 T cell population in islets and blood of NOD mice that is similar in size to the population identified with the tetramer loaded with the NRP-V7 mimic peptide (44). Similarly, the BDC-13 tetramer used here yielded brighter staining and labeled a larger population of cells than the BDC-11 tetramer with the shorter peptide as well as the BDC-Osa tetramer. Therefore, it is possible that CD4 T cell populations could also be detected in the thymus for some of the other peptides investigated here, provided that the peptide sequences are optimized in such a fashion. A potential limitation of the peptide mimetic approach is that not all T cells labeled with such a tetramer may recognize the native peptide, and this issue can in part be addressed by use of two or more mimetics that stimulate the same T cell population.

MHC class II tetramers have been generated by covalent attachment of the peptide of interest to the N terminus of the  $\beta$ -chain, but this approach requires the generation of a new recombinant virus/transfectant for every peptide (19). Generation of tetramers from MHC class II/CLIP precursors offers several significant advantages: 1) many different tetramers can be generated from the same MHC class II/CLIP precursor, permitting investigation of a number of different candidate peptides. This aspect is critical because the key T cell epitopes are not known with certainty in any autoimmune disease, except for animal models where disease is induced by deliberate immunization with a single Ag or peptide; 2) peptide variants that increase the affinity to the MHC molecule or the TCR can be investigated. The length of the peptide can also be optimized, an aspect that may be significant for peptides that have the potential to bind to the MHC class II molecule in more than one register; 3) binding occurs at the acidic pH of the endosomal compartment, rather than the neutral pH of the endoplasmic reticulum. This aspect may be relevant in select cases where different binding registers of the same peptides are possible and where the protonation state of the peptide or the MHC molecule may determine the preferred binding mode. Affinity purification of the final complex may not always be necessary, due to the high peptide loading efficiencies observed here. Nevertheless, this step can be

used to confirm appropriate loading and to exclude the possibility that staining is due to a contaminating peptide. The peptide exchange procedure may also permit tetramers of DQ8/peptide complexes to be generated for the analysis of islet-specific T cells in the human disease. Empty DQ8 molecules have a strong tendency to aggregate (our unpublished data), indicating that protection of the peptide binding site by an exchangeable, low affinity peptide will be required for the generation of DQ8 tetramers by peptide loading.

These findings have important implications for thymic T cell repertoire development, in particular in terms of MHC-linked susceptibility to autoimmunity. The surprisingly high frequency of CD4 T cells identified with I-A<sup>B7</sup>/BDC tetramers demonstrates that the T cell repertoire in NOD mice can be highly biased, apparently because positive selection of this population is efficient while negative selection is either inefficient or largely absent. An important role of thymic repertoire selection in susceptibility to autoimmunity could explain the exquisite allele specificity observed for disease-associated vs nonassociated MHC class II alleles. A key aspect of MHC-associated susceptibility to type 1 diabetes is the presence of a nonaspartic acid residue at position 57 of both DQ and I-A  $\beta$  chains (5, 6). Based on these data, we propose that MHC class II molecules which confer susceptibility to type 1 diabetes act at two distinct sites: initially in the thymus by promoting efficient positive selection of potentially pathogenic T cell populations and later in pancreatic lymph nodes and islets by presenting islet-derived peptides that induce differentiation of these T cells into effector cells that initiate and propagate the inflammatory process. The stringent structural requirements for peptide presentation implied by the genetic data could thus be explained by the requirement for presentation of different peptides in the thymus and the periphery to the same T cell population. This two-stage model of MHC-linked susceptibility could thus explain the observation that particular structural properties of I-A<sup>B7</sup> and DQ8 are tied to disease susceptibility. In most other DQ and I-A molecules, the aspartic acid residue present at  $\beta$ 57 forms a salt bridge with arginine  $\alpha$ 76, but this salt-bridge is not formed in DQ8 and I-A<sup>B7</sup>. Arginine  $\alpha$ 76 is instead available to form a salt bridge with acidic peptide side chains bound in the P9 pocket (3, 8–10). The  $\beta$ 57 polymorphism may thus permit presentation of positively selecting peptides (with an acidic residue at P9) and simultaneously prevent binding of peptides that could induce negative selection of relevant T cell populations (peptides with side chains that cannot be accommodated in the P9 pocket). Experiments in transgenic NOD mice support this hypothesis because mice that coexpressed a mutant I-A<sup>B7</sup>  $\beta$ -chain with substitutions of residues  $\beta$ 56 and 57 of the P9 pocket or other I-A molecules were protected from the disease (14, 45, 47).

Several other lines of evidence indicate that thymic repertoire selection is critical in the development of type 1 diabetes. In humans, susceptibility to the disease is influenced by the promoter region of the insulin gene (*IDDM2* locus) and protective alleles are associated with higher levels of insulin mRNA in the thymus (48, 49). In NOD mice, a defect in thymic negative selection has been reported. Kishimoto and Sprent (50) demonstrated that negative selection in NOD mice was impaired for a population of semimature thymocytes in the medulla with a CD4<sup>+</sup>CD8<sup>+</sup>HSA<sup>high</sup> phenotype (50). Reduced levels of apoptosis were observed for this cell population in vitro following stimulation with anti-CD3 or anti-CD3 plus anti-CD28 or in vivo following injection of the superantigen staphylococcus enterotoxin B. This defect in apoptosis was not observed in NOR, B6.H-2<sup>B7</sup> or (B6.H-2<sup>B7</sup>  $\times$  NOD)<sub>F1</sub> mice. Lesage et al. (51) demonstrated a T cell intrinsic defect in thymic-negative selection in NOD mice based on a transgenic

model in which a membrane-bound form of hen egg lysozyme (HEL) was expressed in islets, along with a HEL-specific TCR. Negative selection of HEL-specific T cells was defective on the NOD but not the B10 background, and experiments in bone marrow chimeras demonstrated that the defect was T cell intrinsic.

A failure of negative selection has also been implicated for the immunodominant T cell epitope of myelin proteolipid protein (PLP, residues 139–151) in SJL mice. Immunization with this peptide induces a severe, chronic form of experimental autoimmune encephalomyelitis. Only an alternatively spliced form that did not include the exon encoding the PLP (139–151) epitope was detected in the thymus, while both splicing variants were expressed in the target organ (52, 53). This failure of negative selection is evidenced by the fact that PLP (139–151)-specific T cells can be readily detected in nonimmunized mice in a T cell proliferation assay (52). It is possible that the same mechanism is responsible for the observation that T cells recognized by I-A<sup>B7</sup>/BDC tetramers are not deleted in the thymus. MHC class II molecules that confer susceptibility to an autoimmune disease may thus set the stage for disease development by permitting the emergence of potentially pathogenic T cell populations from the thymus.

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